



# *The Journal of Laboratory and Clinical Medicine*

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# The Journal of Laboratory and Clinical Medicine

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# THE BIOLOGIC HALF PERIODS OF SODIUM IN NORMAL MAN IN PATIENTS WITH CONGESTIVE HEART FAILURE, AND IN PATIENTS WITH THE NEPHROTIC SYNDROME AS DETERMINED BY $Na^{22}$ AS THE TRACER

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A substance introduced into an organism tends to be eliminated exponentially; that is the amount excreted is a fairly constant percentage of that which remains. Mathematically the substance is never completely eliminated, and it is therefore preferable to measure the interval of time required to eliminate one half the material introduced; this is known as the *biologic half life period* of the substance. It is important to know this measurement for several reasons:

(1) The length of time required to excrete one half an isotope or labeled substance introduced into an organism is equal to the time required to excrete one half the total amount of the regular form of the substance already present in the organism. Therefore a measurement of the biologic half life period of the substance introduced indicates the total rate of turnover of the regular substance.

(2) Whenever radioelements are introduced into an organism the *safety factor* is determined in part by the time interval of exposure of the organism to radiations from the radioelement. Radioelements with a long physical half life such as  $Na^{22}$  or  $C^{14}$ , will not decay rapidly enough to change their activity appreciably. The degree of their action upon the organism on the other hand is reduced as the radioelement is excreted. Therefore from a knowledge of the biologic half life period the duration of exposure of the organism to the radiations of the substance is known.

(3) *Safety measures* of public health importance are concerned with the biologic half life period of radioelements, and these problems will assume greater import as radioelements are more widely employed.

(4) The biologic half life period of a substance influences the *nature of the study* concerned with labeling by radioactive substances, such as the rate of collection of samples of biologic fluids, the time at which animals must be sacrificed, and clinical management of human subjects. For example, a radioelement with a relatively long biologic half life period and a very short physical half life is not suitable for tracer studies involving over all turnover in the organism.

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(5) The biologic half-life period is an important factor in calculating *dosages* of radioelements to insure levels of counts which permit accuracy of measurements

(6) The influence of *disease* upon the biologic half-life is significant from the point of view of understanding disease and of planning further investigations

(7) This measurement is also valuable in clarifying problems related to *therapy*, characteristics of species in animals and plants, toxicology, and many other problems peculiar to individual experiments

The wide variations in metabolic activity, diet, and general biologic processes affect the biologic half-life of radioelements considerably. This is true in health and particularly in disease. For this reason, values for the biologic half-life of a substance for a normal organism cannot be applied empirically to an abnormal one. For example, since the excretion of a substance varies with each subject and with the intake of that substance by the organism, any interpretation based upon an average biologic half-life period is only an approximation for some other subject with a different intake and a different biologic state.

During the course of the study of the rate of sodium turnover in normal human subjects and the influence of congestive heart failure on sodium excretion with a limited supply of long half-life radiosodium ( $\text{Na}^{22}$  with  $t_{1/2}$  of three years), sufficient data were obtained to indicate the biologic half-life period of sodium in man. These help elucidate the influences of diet and drugs upon the biologic half-life in normal man as well as in patients with chronic congestive heart failure and in those with the nephrotic syndrome of chronic glomerulonephritis.

#### MATERIALS AND METHODS

In the studies with  $\text{Na}^{22}$  not designed primarily for measuring the biologic half-life period of sodium, twelve subjects were observed continuously for periods varying from twenty to seventy days. Four of these were normal subjects, six had chronic congestive heart failure (two were slowly improving, two rapidly improving, and two slowly becoming worse), and two patients had the nephrotic syndrome of chronic glomerulonephritis (see Table I for clinical details).

$\text{Na}^{22}$ , as  $\text{NaCl}$  in approximately 2 c.c. of water, was administered intravenously to each subject. Doses with an activity such that there were 17,725,000 counts per minute (about 09 mc) were administered to seven subjects (Nos. 2, 3, 4, 5, 6, 11, and 12), 12,500,000 counts per minute (about 06 mc) to three subjects (Nos. 1, 7, and 9), and 10,000,000 counts per minute (about 05 mc) to two subjects (Nos. 8 and 10). It was found that sufficiently high counts could be obtained by injection of smaller doses, necessitated by the limited supply of  $\text{Na}^{22}$ .

Each specimen of urine during the entire period of study was collected separately, and daily blood samples were taken. The volume of each urine specimen, and the radioactive count, were recorded so that total elimination of radiosodium could be determined. Counts of blood serum were followed as an index of radiosodium concentration in the extracellular fluid.

Sixteen free falling drops of urine or serum from a calibrated micropipette were placed on filter paper disks fixed with rubber cement to metal disks so that the quantity and geometric characteristics of each sample remained constant. The samples were counted for

TABLE I. CLINICAL DATA

SUBJECT NO	AGE (YR)	SEX	INITIAL WEIGHT (POUNDS)	DIAGNOSIS
<i>A Normal or Control</i>				
1	11	M	112	Obliterative pleuritis
2	16	F	124	Acute rheumatic fever
	"	F	121	Esophageal peptic ulcer
4	29	F	122	Duodenal ulcer
<i>B Congestive Heart Failure (Slowly Improving)</i>				
5	17	F	152	Hypertension
6	18	F	162	Arterial hypertension
<i>C Congestive Heart Failure (Rapidly Improving)</i>				
7	6	M	115	Hypertension
8	17	F	124	Rheumatic heart disease (mitral) atrial fibrillation
<i>D Congestive Heart Failure (Slowly Becoming Worse)</i>				
9	16	M	155.5	Syphilitic aortic insufficiency
10	54	F	129.5	Hypertension
<i>E Chronic Hemorrhagic Nephritis (Nephrotic Syndrome)</i>				
11	15	F	138	Renal function 25 to 30 per cent normal slowly improv- ing
12	28	F	282.10	Renal function 20 to 30 per cent normal

five minutes with men window Geiger Muller counters. Corrections for background were made, and the data were recorded in counts per minute per cubic centimeter of fluid. In order to compare the concentrations of radiosodium in the serum, all counts were corrected to correspond to an injected dose of 17,750,000 counts per minute for each subject. The range of error was plus minus 3 per cent.

The urinary excretion of Na was expressed in terms of the percentage of injected Na which was not eliminated by the kidneys. This value, represented symbolically as %N<sub>t</sub>, was calculated from the equation

$$\%N_t = \left( 1 - \frac{\sum k}{N} \right) \times 100$$

where

N = injected Na in counts per minute

k = Na excreted in urine only during the s<sup>th</sup> day after injection expressed in counts per minute

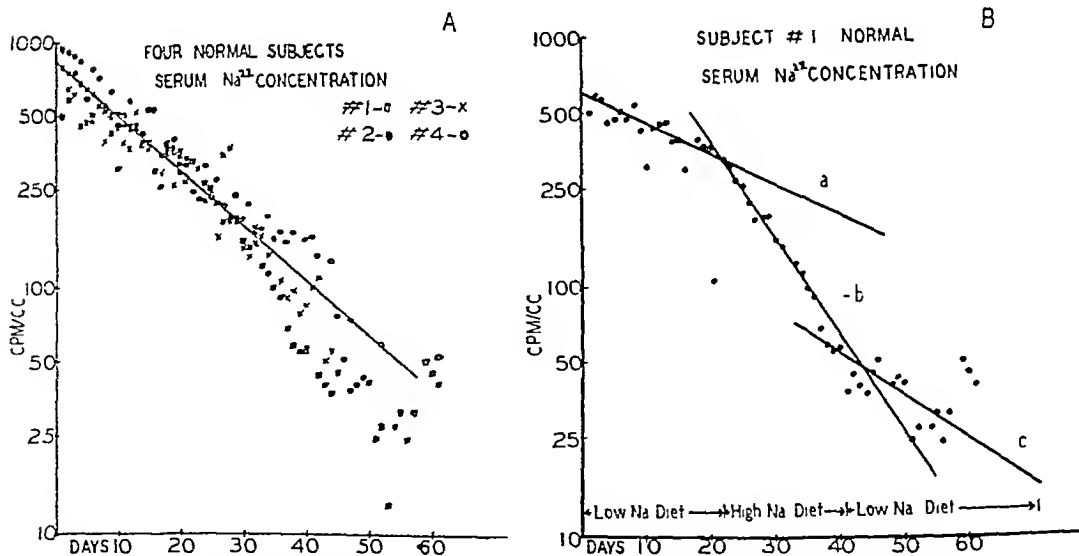
%N<sub>t</sub> = percentage of injected Na not excreted in urine by the end of the t<sup>th</sup> day after injection

Weights and fluid intake and output were recorded daily for each subject. The sodium intake was varied in some instances from low (<17 Gm NaCl per day) to regular (8 Gm NaCl per day), to high (137 Gm NaCl per day), and the effect on the rate of elimination of radiosodium was noted. Mercurial diuretics\* and other drugs, used frequently in both the diseased and normal subjects, exerted some influence on the rate of excretion of the radio sodium.

\*Mercurhydrin (sodium salt of methoxyoximercuripropylsuccinylurea with theophylline) furnished by courtesy of Lakeside Laboratories Milwaukee Wis.

TABLE II THE INDIVIDUAL AND MEAN  $C_{1/2}$  AND  $U_{1/2}$  VALUES FOR THE SUBJECTS STUDIED

SUBJECT NO	$C_{1/2}$	$U_{1/2}$	DAYS OF CONTINUOUS OBSERVATION	WEIGHT CHANGE (POUNDS)
<i>I Normal or Control</i>				
1	14	30	62	-3.5
2	13	9	22	-14
3	12	42	45	-11
4	14	34	65	2.25
Mean	13.3	28.8	48.5	-6.6
<i>B Congestive Heart Failure (Slowly Improving)</i>				
5	40	60	35	-18
6	12	72	46	-7
Mean	41	66	40.5	-12.5
<i>C Congestive Heart Failure (Rapidly Improving)</i>				
7	13	26	62	-29
8	28	33	58	-17
Mean	20.5	29.5	60	-23
<i>D Congestive Heart Failure (Slowly Becoming Worse)</i>				
9	24	72	68	17
10	30	48	58	-5.5
Mean	27	60	63	5.75
<i>E Chronic Hemorrhagic Nephritis (Slowly Improving)</i>				
11	58	660	45	15
12	54	366	71	-86
Mean	56	513	58	-35.5

Fig 1—Semilogarithmic graphs of relation of changes in serum  $Na^{22}$  concentration (counts per minute per cubic centimeter) to time

A, Four subjects without cardiovascular disease. The mean rate of fall in concentration was such that half-concentration was reached in 13.3 days ( $C_{1/2}$ ).

B, Normal Subject No 1, showing a change in rate of fall in concentration with variations in sodium content of the diet: low sodium diet (17 Gm NaCl daily) and high sodium diet (137 Gm NaCl daily). At rate a, with low sodium diet serum concentration reached half the initial value in twenty-five days. At rate b with a high sodium diet half serum concentration was reached in eight days and at rate c when a low sodium diet was resumed half-concentration was reached in eighteen days.

## RESULTS

Results are summarized in Table II and in Figs 1 2 3 4 and 5

*1 Controls*—In the four control subjects who had no disturbance in the cardiovascular system and no edema the serum concentration of  $\text{Na}^+$  dropped to half the initial level in an average of 13.3 days, the extremes being twelve and fourteen days (Table II and Figs 1 and 4). The rate of elimination of the isotope in the urine was such that one half of the  $\text{Na}^+$  administered would have been excreted in an average of 28.8 days with a range of nine and forty-two days (Table II and Figs 3 and 4). Subject No. 1 demonstrated the influence which intake of sodium chloride has on the rate of elimination of  $\text{Na}^+$  from the body (Figs 1, B and 3, B). The rate of decline in serum concentration whereby one half the initial concentration was reached in twenty-five days when the patient was given 17 Gm. of available sodium chloride increased to a rate whereby one half the initial concentration was attained in eight days (three times as rapid) when 137 Gm. of available chloride was allowed and then changed again to a reduced rate of eighteen days when the patient again received 17 Gm. available sodium chloride (Fig. 1, B). Similar response was noted for rates of elimination in the urine (Fig. 3, B).

*2 Patients With Chronic Congestive Heart Failure —*

*(a) Patients Slowly Improving* In two patients who were slowly recovering from congestive heart failure forty and forty-two days were required for the serum concentration of  $\text{Na}^+$  to reach one half the initial level (Table II and Figs 2, A and 4). These patients required about three times as many days as the control subjects for reduction of serum concentration of  $\text{Na}^+$  to half the initial level. The  $\text{Na}^+$  was excreted in the urine at such a rate that sixty and seventy-two days would have been required to excrete one half the administered  $\text{Na}^+$  (Table II and Figs 3, C and 4). The rate of excretion of  $\text{Na}^+$  in the urine of the control subjects was more than twice as rapid as in the patients with heart failure.

*(b) Patients Rapidly Improving* The two patients with chronic congestive heart failure who improved rapidly required an average of 20.5 days (extremes, thirteen and twenty-eight days) for the serum concentration of  $\text{Na}^{22}$  to reach one half the initial level (Table II and Figs 2, B and 4). The mean rate of excretion of  $\text{Na}^{22}$  was 29.5 days, the extremes being twenty-six and thirty-three days (Table II and Figs 3, D and 4). Although the average rate of decline in the serum concentration in Patient No. 7 was thirteen days, once improvement began, the rate of decline exceeded that of the control subjects. Initially the rate of decrease in blood concentration of  $\text{Na}^+$  was such that half concentration would have been reached in fifty-five days. During rapid improvement however this level was attained in six days, a rate more rapid even than that of the control subject on a high sodium intake. The rates of elimination of  $\text{Na}^+$  in the urine of this patient tended to parallel changes in concentration in the serum (Figs 2, B and 3, D). Patient No. 8 also showed two rates of decline in serum concentration of  $\text{Na}^{22}$  (seventy-one and twenty-four days respectively required to reach

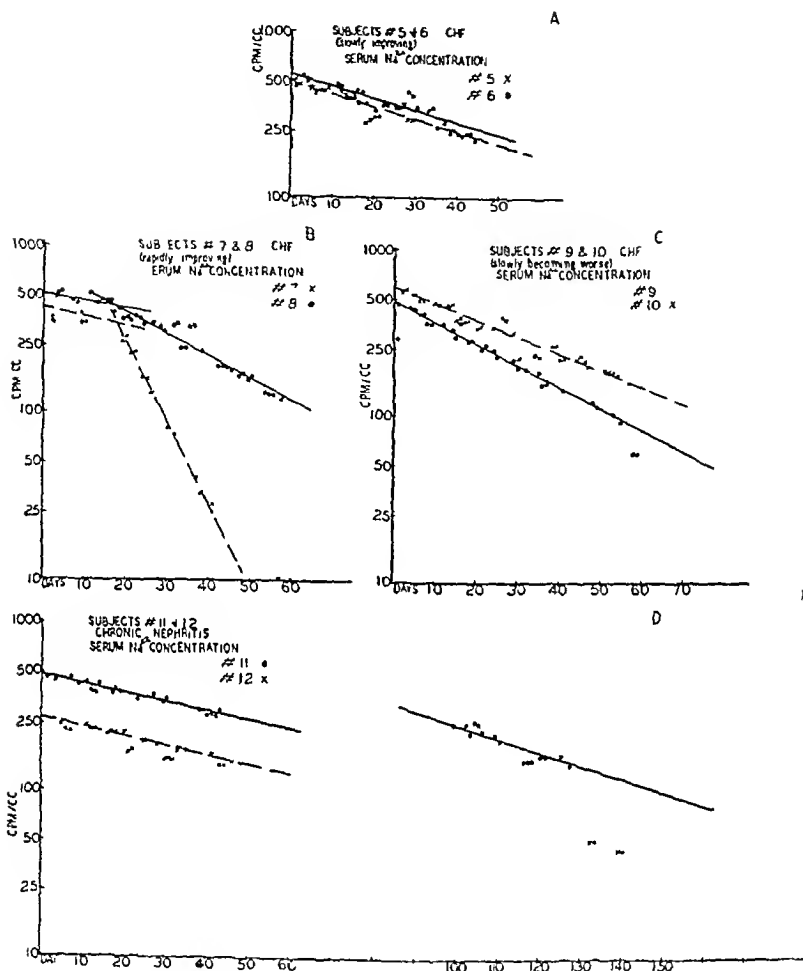


Fig 2—Semilogarithmic graphs of changes in serum  $\text{Na}^{22}$  concentration (counts per minute per cubic centimeter) as a function of the time for six patients with congestive heart failure and two patients with the nephrotic syndrome of chronic glomerulonephritis

A, Two patients with congestive heart failure who were slowly improving. Patient No 5 showed a mean rate of fall in serum  $\text{Na}^{22}$  concentration so that half-concentration was reached in forty days ( $C_{1/2}$ ). Patient No 6 showed a mean rate of fall in concentration so that half-concentration was reached in forty-two days. It may be noted on the graphs that for each of these patients several rates of change existed, although only the mean rate is shown by the straight line.

B, Two patients with congestive heart failure who were rapidly improving. Patient No 7 showed two distinct rates of fall in serum  $\text{Na}^{22}$  concentration. The first rate maintained for eighteen days was such that half-concentration would have been reached in six days. Patient No 8 also showed two distinct rates of fall in serum  $\text{Na}^{22}$  concentrations. The first rate present for eighteen days was such that half-concentration would have been reached in seventy-one days whereas with the second rate twenty-four days would have been necessary.

C, Two patients with congestive heart failure who were slowly becoming worse. Patient No 9 showed a mean rate of fall in serum  $\text{Na}^{22}$  concentration so that half-concentration was reached in twenty-four days ( $C_{1/2}$ ). Patient No 10 showed a mean rate of fall in concentration so that half-concentration was reached in thirty days. Several rates of change in concentration may be noted although only the mean rate for each patient is indicated by the straight line.

D, Two patients with the nephrotic syndrome of chronic glomerulonephritis. Both of these patients were discharged from the hospital and later readmitted for continuation of the studies. On the first admission Patient No 11 showed a mean rate of fall in serum  $\text{Na}^{22}$  concentration so that half-concentration would have been reached in fifty-eight days ( $C_{1/2}$ ). During the second period of study this patient showed a mean rate of fall in concentration so that half-concentration would have been reached in thirty-seven days and Patient No 12 required fifty-four days.

half concentration) and at least two rates of elimination of Na in the urine (Figs 2, B and 3, D) These two patients eliminated Na more rapidly than the two patients who were improving slowly

(c) *Patients Slowly Becoming Worse* Two patients gradually became clinically worse throughout the period of study The serum concentration of Na was reduced to one half the initial value in an average of twenty seven days the extremes being twenty four and thirty days (Table II and Figs 2 C and 4) The mean rate of loss of Na in the urine was such that one half the administered Na would have been excreted in sixty days, with extremes being forty eight and seventy two days (Table II and Fig 3, F) These patients required a longer period of time to excrete the Na than did the control subjects or the patients with congestive heart failure who were rapidly improving but essentially the same time was necessary in patients who were slowly improving

3 *Patients With the Nephrotic Syndrome of Chronic Glomerulonephritis* — The two patients with the nephrotic syndrome showed the slowest rates of elimination of Na The decline in the serum concentration of Na was such that an average of fifty six days (extremes fifty four and fifty eight) would have been required for the serum level to reach one half the initial value (Table II and Figs 2, D and 4) The rate of Na excretion in the urine was extremely slow in both patients an average of 513 days would have been necessary to eliminate one half the Na administered (Table II and Fig 3, F) The control subjects exhibited a rate of decline in the serum concentration of Na<sup>22</sup> more than four times as rapid as in these patients and the rate of urinary elimination was ten to twenty times as rapid (Table II and Fig 4)

#### DISCUSSION

Morgan<sup>1</sup> suggested the symbol "Te" for the body elimination half life This term is satisfactory if the time required to eliminate one half the radioactive material administered can be determined without too much difficulty Unfortunately, this is not easily accomplished in man, especially for sodium Results indicate that there are considerable variations in normal as well as in diseased man Because of these wide variations from moment to moment and because of differing conditions of environment, diet physical activity, severity stage and course of disease, therapy, previous physiologic state and many other factors, it is possible to obtain only an approximation of the time required to eliminate one half the radioactive material administered Furthermore values observed for changes in blood concentration differ from those for elimination in the urine (Table II Figs 1 to 4) It is, therefore, preferable to indicate in the symbol for the half elimination time the method by which the value was obtained It is suggested that the following symbols be employed in the discussion of rates of elimination

$B_{1/2}$  = *biologic half life*, that is the time required to eliminate one half the administered tracer substance from the body This corresponds to the "Te" value of Morgan

$C_{1/2}$  = *concentration one half*, that is the length of time required for the concentration of the tracer material in the body fluid or substance or



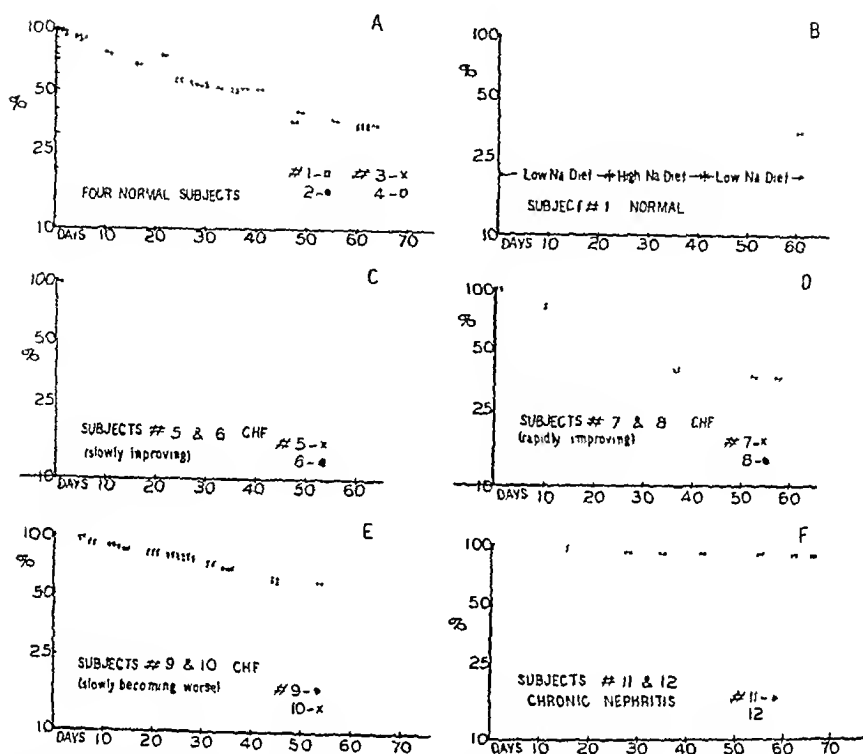
URINARY EXCRETION OF  $\text{Na}^{22}$ 

Fig 3—Semilogarithmic graphs for all subjects showing the urinary excretion of  $\text{Na}^{22}$  (percentage of injected  $\text{Na}^{22}$  which was not eliminated by the urine). This value was obtained from the total counts of  $\text{Na}^{22}$  excreted daily through the urine. That for the first day was subtracted from the total counts injected. The total for each successive day was subtracted values represent that radiosodium which has remained within the body plus that which has been excreted by some other route i.e. that  $\text{Na}^{22}$  which has not been eliminated in the urine. This also indicates the rate of excretion by way of the urine.

A, Four subjects without cardiovascular disease. If all the  $\text{Na}^{22}$  were eliminated in the urine the day upon which one-half of the injected radiosodium would have been excreted (mean value  $U_1$ ).

B, Normal Subject No. 1 shows changes in rate of excretion of  $\text{Na}^{22}$  in the urine with changes in the sodium content of the diet. For twenty-two days during a low sodium diet the rate of elimination in the urine was such that one-half the sodium present in the body would have been eliminated in one hundred days ( $U_1$ ). For the next nineteen days during a high sodium diet the rate of excretion increased so that one-half the body sodium would have been excreted in the urine in nineteen days. For the last nineteen days of observation during a low sodium diet and administration of antidiuretics the rate of urinary excretion of body sodium was such that 250 days would have been required for excretion of one-half the sodium present at the beginning of that period. Actually one-half the injected  $\text{Na}^{22}$  was excreted in thirty days.

C, Two patients with congestive heart failure who were slowly improving. Several different rates of excretion for each patient may be noted. The mean rate for the two patients being sixty-six days for excretion of half the  $\text{Na}^{22}$  injected.

D, Two patients with congestive heart failure who were rapidly improving. Several rates of excretion may be observed. The mean length of time required to excrete one-half the injected  $\text{Na}^{22}$  by the urine was 29.5 days ( $U_1$ ).

E, Two patients with congestive heart failure who were slowly becoming worse. Changes in rate of excretion may be noted as in the other subjects. The mean time necessary for excretion of one-half the injected sodium through the urine was sixty days.

F, Two patients with the nephrotic syndrome of chronic glomerulonephritis. If sodium were excreted only by the urine a mean of 513 days would have been required to excrete one-half the injected radiosodium.

specific compartment to reach one half the concentration existing at any time after equilibrium of distribution of the tracer has been reached. It is thus possible to speak of the  $C_1$  for cerebrospinal fluid,  $C_{12}$  for hepatic parenchyma,  $C_1$  for blood serum.

$U_{1/2}$  = *urinary elimination one half* that is the length of time necessary to eliminate in the urine one half the tracer material administered.

From the point of view of *safety*<sup>1</sup> it is actually the  $C_1$  that is important. Since the  $C_{1/2}$  and  $U_{1/2}$  are sometimes extremely different, particularly in abnormal states (Table II, Lines 2, 3 and 4), the  $U_{1/2}$  value is of little assistance in calculation of dosages and may result in error.  $B_{1/2}$  is certainly important but is not easily determined in man especially for sodium. Such a determination would require measuring all of the radiosodium excreted by *all* avenues for long periods in a great many persons under varying conditions of diet, environment, disease, and therapy; this is a tremendous task. With the present state of knowledge of radioelements and biologic influences of radioactivity,  $C_{1/2}$  and  $U_{1/2}$  furnish important data of useful physiologic nature. The possibilities of variations in  $B_1$ ,  $C_1$ , and  $U_{1/2}$  are discussed in a second paper, and various mechanisms by which they may differ considerably are suggested.

In all subjects studied  $C_{1/2}$  was less than  $U_{1/2}$  except in Control Subject No. 2. Careful analysis of the data failed to suggest a rational explanation for this discrepancy.  $U_{1/2}$  is expected to be greater than  $C_{1/2}$  since sodium is eliminated by avenues other than the urine such as sweat, tears, sputum, feces, vomitus, and other body fluids. "Binding" of sodium in cells and onto cells, proteins, and other complex molecules can account in part for these differences. These factors should be studied in experiments designed to determine the  $B_1$ ; the present experiments however were planned for other purposes and it was not possible to make these measurements. They were satisfactory for determining the  $C_{1/2}$  and  $U_{1/2}$  in the subjects observed.

The  $C_1$  and  $B_{1/2}$  would be expected to be identical in subjects in whom the size of the sodium compartment fails to change, and this is more likely to occur in normal than in diseased subjects. This is probably true for Control Subject No. 1, who was apparently normal in every respect. This was true clinically for Subject No. 2 except that she had fever with arthralgia three weeks before initiation of the studies. Control Subjects Nos. 3 and 4, who suffered from dyspepsia from time to time and experienced vomiting occasionally, were not truly normal but could be considered so for practical purposes.

It is obvious from the values obtained for  $C_{1/2}$  and  $U_{1/2}$  that  $Na^{24}$  is not suitable for measuring these parameters or for measuring  $B_{1/2}$ . Na on the other hand, is excellent for such purposes. Although the supply of Na was limited, a relatively large number of subjects was observed for long periods of time. If the  $C_{1/2}$  values approximate the  $B_{1/2}$ , the physical half life period (three years) of Na is of little value in safety considerations for the subject, because the radiosodium is excreted long before sufficient physical decay can occur, that is for biologic purposes,  $Na^{24}$  may be considered stable.  $Na^{24}$ , on the other hand, rapidly decays to levels of radioactivity too low for accurate measurements of biologic decay periods.

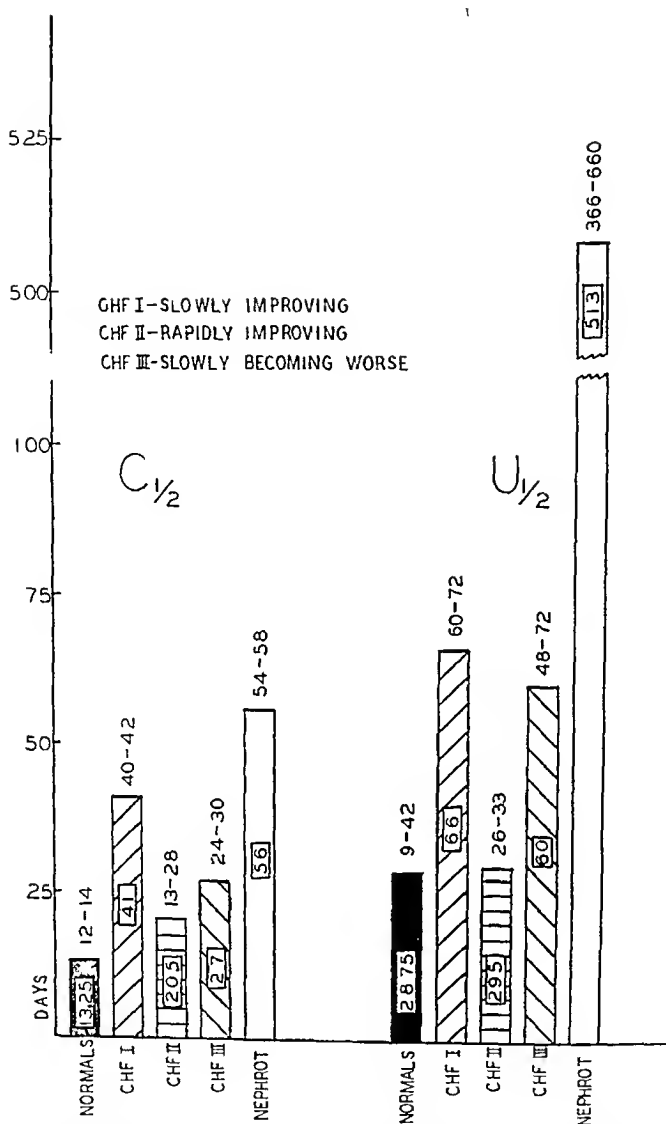


Fig. 4—A bar diagram of the mean  $C_{1/2}$  (value of time days) for the serum  $Na^{22}$  concentration to reach one-half the initial equilibrium concentration ( $C_{1/2}$ ) and the mean time for one-half the injected  $Na^{22}$  to be excreted in the urine ( $U_{1/2}$ ). The mean value for each group is enclosed in the column and the extremes are indicated above the column.

Although the subjects were followed for as many as sixty consecutive days, the  $C_{1/2}$  or  $U_{1/2}$  values often exceeded this period considerably, so that it was necessary to extrapolate the observed curves to obtain the values of  $C_{1/2}$  and  $U_{1/2}$  recorded. This was true for only two of the  $C_{1/2}$  determinations and for seven of the  $U_{1/2}$ . Therefore, all the values of  $C_{1/2}$  and  $U_{1/2}$  actually indicated the rates of change that existed only for the days observed.

A number of factors other than excretion can alter the  $C_{1/2}$ . As mentioned previously, a fixation of sodium to cells or protein and other large molecules will reduce the sodium content of the serum and the  $Na^{22}$  concentration. Fixa-

tion of binding of the tracer can result in a state such that  $C_{1/2}$  cannot be equal to  $B_{1/2}$ . Another important factor is a discrepancy in water intake and output particularly in states of edema. When more water is taken in than is excreted

RELATION OF DIET TO CHANGES IN RATES OF EXCRETION AND OF DECREASE IN SERUM CONCENTRATION OF  $Na^+$

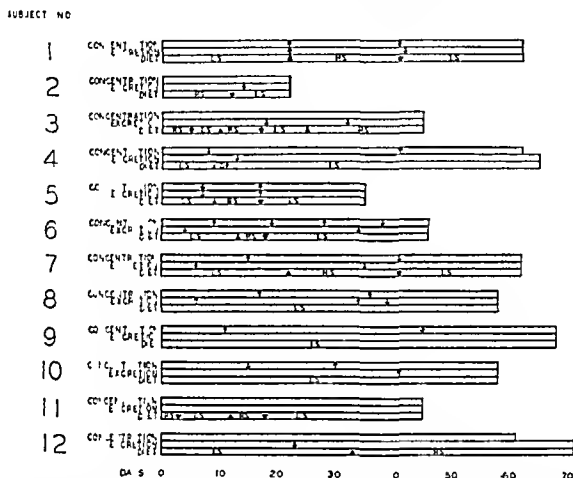


FIG. 5—The relationship of change in sodium content of the diet and change in the rate of decrease of serum  $Na^+$  concentration and change in the rate of urinary excretion of  $Na^+$  for each subject. Sodium content of the diet is indicated by PS (high sodium 13.7 Gm NaCl daily), LS (low sodium less than 1 Gm NaCl daily), PS (regular sodium intake 8 Gm daily). Changes in rates are denoted by arrow. ↑ indicates a more rapid rate of change in serum  $Na^+$  concentration or rate of urinary excretion. ↓ indicates a reduction in either rate. Time is recorded as days of observation. High correlation of change in diet and change in rate may be noted in Subject No. 1 but poor correlation exists in the others (see text).

then the  $Na^+$  content of the body fluids will be diluted and the serum  $Na^+$  concentration will be diminished. If  $Na^2$  counts for the serum had been employed alone in the interpretation the rate of excretion of  $Na^2$  so obtained would have been inaccurate. In fact, if the sodium excretion fell almost to zero in a man who rapidly became edematous shortly after administration of a tracer dose of  $Na^{22}$  and if the edema were sufficiently severe to double the volume of the sodium compartment of the body then the  $Na^2$  concentration in the serum would be reduced to one half the initial value. Thus the subject might attain a  $C_{1/2}$  value without excreting any sodium but such a value would certainly differ greatly from the  $B_{1/2}$ . This dilution phenomenon was noted in some of the edematous patients studied (Subjects No. 9, 11 and 12).

If the reverse situation occurs that is if an intensely edematous subject loses water more rapidly than sodium, then an actual rise in the serum concentration of  $Na^+$  and therefore in the  $C_{1/2}$  time would ensue. Here too the

$C_{1/2}$  and  $B_{1/2}$  values would differ. It is obvious then, that the  $C_{1/2}$  and  $B_{1/2}$  values cannot be accepted as similar, particularly in subjects with edematous states.

A glance at the biologic decay curves reveals considerable fluctuation in the serum concentration of  $\text{Na}^{22}$ . These fluctuations are partially due to technical variations but in greater part are attributable to changes produced by phenomena described in the preceding paragraph. Furthermore, some of the variations are the result of responses to drugs and other pharmacologic reactions. The responses to such materials as desoxycorticosterone acetate, mercurial diuretic, sodium chloride and water intake, betahypophosphamine, and ammonium chloride were studied. These substances definitely influenced the  $C_{1/2}$ ,  $U_{1/2}$  and  $B_{1/2}$  of the subjects. In some instances these correlations were difficult to interpret whereas in others they were easy. For example, in Subject No. 1, the  $C_{1/2}$  and  $U_{1/2}$  were influenced significantly by the sodium intake in the diet, a correlation made possible by allowing mainly dietary sodium to vary (Figs 1 B and 3 B). When the subject was on a low sodium chloride diet (17 Gm of available NaCl),  $C_{1/2}$  and  $U_{1/2}$  were definitely longer than during the interval when he was receiving relatively large quantities of sodium (137 Gm of available NaCl). For example, initially while on a low sodium intake,  $C_{1/2}$  and  $U_{1/2}$  values were twenty-five and one hundred days respectively, on high sodium intake they were reduced to eight and nineteen days, and when a low sodium intake was resumed, the  $C_{1/2}$  and  $U_{1/2}$  values lengthened to eighteen and two hundred and fifty days respectively. The correlations were impressive and, of course, predictable. Most subjects, however, failed to show a definite relationship between  $C_{1/2}$  and  $U_{1/2}$  and sodium intake (Fig 5). This failure is related to other factors such as drugs, water intake, and the like, which influence  $C_{1/2}$  and  $U_{1/2}$ .

Another obvious factor of importance to consider when measuring the  $C_{1/2}$  and  $B_{1/2}$  in edematous subjects is that concerned with the mechanical removal of fluids from various compartments of the body. For example, if large quantities of ascitic fluid or pleural fluid are removed from a subject at equilibrium and containing  $\text{Na}^{22}$ ,  $B_{1/2}$  will be changed without changing  $C_{1/2}$ . This factor may become significant in a subject who is receiving repeated paracenteses. Proper considerations can be made for such factors.

Extremely edematous subjects who rapidly improve may excrete sodium at a rate considerably greater than that observed in the normal subject under the same therapeutic regimen. This phenomenon was noted for Subject No. 7, who had severe chronic congestive heart failure with anasarca. Diuresis was intense and prolonged, and during this period the  $C_{1/2}$  and  $U_{1/2}$  were six and twenty-two days respectively. The  $C_{1/2}$  was shorter than that observed for the control subjects, even when sodium intake was high. Obviously, the subject was excreting the fluid of edema and  $\text{Na}^{22}$  at a rapid rate, with the  $\text{Na}^{22}$  elimination out of proportion to the water. This resulted in a relatively short  $C_{1/2}$ . At the same time the  $B_{1/2}$  period would have to be short.

The results demonstrate reduction of sodium excretion in patients with chronic congestive heart failure and chronic glomerulonephritis of the nephrotic type. The mean  $C_{1/2}$  periods were several times longer for these diseased sub-

jects than for the controls. There were periods of improvement however for patients with congestive heart failure when  $C_{1/2}$  and  $U_{1/2}$  were shortened. These observations indicate the importance of considering the abnormal state in calculation of safety doses of radiosodium. It is essential that such variations of  $B_{1/2}$  be known for any type of radioelement.

It is relatively easy to determine  $C_{1/2}$  and  $U_{1/2}$  for a radioelement whereas for a labeled compound or even a simple molecule it is much more difficult. The labeling isotope is being 'turned over' in the molecule of the labeled substance, and the substance itself is always in the process of breakdown and partial or complete resynthesis. Sometimes it is even possible to label complex structures to trace them. But a parameter difficult to evaluate even for elements. This is well exemplified by the measurement of the  $B_{1/2}$  period of erythrocytes with  $\text{Na}^{24}\text{I}$  is not as satisfactory as  $\text{Na}^{24}$  and  $\text{P}^{32}$  is even less satisfactory as a tracer isotope because it does not remain within the erythrocytes throughout their life. Proper consideration must be given such problems in determination of the  $C_{1/2}$ ,  $U_{1/2}$ , or  $B_{1/2}$  periods.

#### SUMMARY

Rates of elimination of sodium were studied with  $\text{Na}^{24}$  as a tracer in normal man and in man suffering from chronic congestive heart failure or from chronic glomerulonephritis of the nephrotic type.

Because of the nature of the experiments the true biologic half life period ( $B_{1/2}$ ) was not measured directly. Instead the time necessary to reduce the serum concentration to one half the initial level after the establishment of equilibrium ( $C_{1/2}$ ) and the time required to eliminate one half the injected  $\text{Na}^{24}$  in the urine ( $U_{1/2}$ ) were determined.  $t_{1/2}$  values obtained were usually less than the  $U_{1/2}$  ones.  $C_{1/2}$  in the control subjects most probably was equal to  $B_{1/2}$  but this was not likely to be true for the abnormal subjects.  $C_{1/2}$  and  $U_{1/2}$  varied considerably with normal physiologic phenomena and with such abnormal states as congestive heart failure, nephrosis, and with administration of drugs, and sodium and water intake. For example a diet low in salt appeared to lengthen the time of  $C_{1/2}$  and  $U_{1/2}$  whereas a diet high in salt was shown to shorten them.

$C_{1/2}$  was increased about threefold in patients with chronic congestive heart failure and about fivefold in the presence of the nephrotic syndrome.  $U_{1/2}$  was increased to an even greater extent by these diseases and was influenced by the progress of the process. When the diseases were aggravated and fluid of edema was accumulating the influence of dilution modified the  $C_{1/2}$ .

The importance of these observations and the relative general significance of  $B_{1/2}$ ,  $C_{1/2}$ , and  $U_{1/2}$  periods in the biologic application of radioelements, particularly radiosodium, have been discussed.

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# THEORETIC CONSIDERATIONS OF BIOLOGIC DECAY RATES OF ISOTOPES

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THE rate with which isotopes are eliminated from an organism is of importance physiologically, in safety considerations, in calculation of dosages, and for many other reasons. In previous studies<sup>†</sup> concerned with biologic decay periods for the radioactive isotope of sodium ( $\text{Na}^{22}$ ), it became necessary to enter into theoretic considerations of rates of change in concentration of the isotope in the body fluids and in other compartments as well as rates of elimination. These considerations hold for any tracer and for the entire organism or any compartment within it, regardless of size or location. Results obtained and concepts developed are worthy of report because of their general application to biologic and other tracer experiments.

After a single administration of an isotope and establishment of homeostasis, elimination and change in concentration of the isotope in the compartments of the body are dependent upon many related factors. Only a few of the factors are relatively fixed, and variations in them may increase with abnormal states. The patterns of elimination and variations in concentration are complex. For example the excretion of an isotope is related to such phenomena as the following:

- 1 There is daily lowering in concentration and/or total amount of the isotope within the body because of continuous excretion. From experimental work done to date, it appears that excretion occurs exponentially.

- 2 Change in concentration of the tracer material results whenever the volume of the isotopic compartment varies.

- 3 The daily intake of the nontracer form of the element usually affects the behavior of the tracer form.

It is impossible to consider all the factors associated with elimination of an isotope or variations in concentrations within its compartments in the body, among those herein considered are:

- 1 Variations in intake of the nontracer form of the substance.

- 2 Variations in rates of elimination of the nontracer substance from the organism.

- 3 Variations in volume of the compartments of the substance.

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These factors deserve attention in biologic work concerned with tracer studies, since any one of them can produce a considerable change in concentration or elimination of the tracer element. For this reason such phenomena which are independent of the physiologic processes being observed must be evaluated adequately or controlled.

It is well to define certain terms employed in this discussion.

*Elimination* denotes any movement of the substance being studied from the compartment under observation. For example, if the sodium in the extracellular fluids passes into cells or into the urine it is considered to have been eliminated from the compartment of extracellular fluid regardless of the mechanism by which this transfer occurs.

*Intake* refers to any addition of the substance to the compartment under study. Thus, sodium added to the extracellular fluid compartment from the diet or from mobilization of sodium from bones or cells is considered intake.

*Volume of the compartment* indicates the volume of that portion of the organism which is under study with the tracer. It is much safer to include in any compartment that portion of the organism which is physiologically (including physical and chemical behavior) homogeneous. For example, it would be less satisfactory to group all portions of the body in which sodium is found as the sodium compartment than it would be to consider separately the sodium compartment of extracellular fluid of bone of cerebrospinal fluid, and so forth. This is particularly true when rapid or sudden changes are occurring in the element being traced.

From the three terms just defined it becomes obvious that it is possible to consider the whole organism as one compartment or any portion of it as a compartment, for example, the extracellular fluid space pleural cavity individual cell, or nucleus of a cell.

#### THE PROBLEM

It is advisable at this moment to present briefly the problem to be considered in these discussions. This can be done satisfactorily through the medium of Fig. 1, which illustrates a tank. At time  $t$  this tank contains a compartment with a volume,  $V_i$  into which is dispersed a substance  $M_i$  in solution or other state, to be observed. To the tank there is being added continuously more of the material which constitutes the dispersing compartment or medium  $V_r$  as well as more of the substance to be observed,  $I$ . Simultaneously, a certain amount of the dispersing medium  $V_k$  and a certain amount of the substance under observation  $K$ , are being removed continuously from the tank through a stopcock. If it is desired to trace this substance within the tank, then at a time  $t = 0$  the known quantity of a tracer form,  $N$ , should be added to the tank. If it is added at a given moment, it soon attains equilibrium of distribution with the nontracer form the substance to be observed. Moreover, it is important to remember that since the tracer form is *not* added continuously, this form  $N$  is being removed *gradually* from the tank by way of the stopcock.



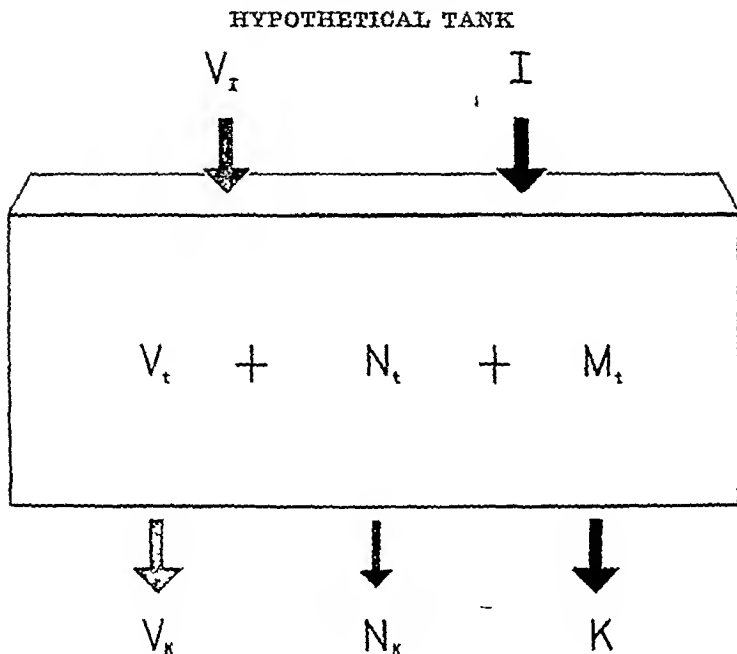


Fig 1—Diagram of a tank or compartment containing  $\text{Na}^{23}$  in water in which the concentration of  $\text{Na}^{23}$  remains constant.  $\text{Na}^{23}$  as a tracer substance is added at an instant to label the  $\text{Na}^{23}$ . The  $\text{Na}^{23}$  and  $\text{Na}^{23}$  are always thoroughly mixed. To this tank more  $\text{Na}^{23}$  and water are being added while  $\text{Na}^{23}$ ,  $\text{Na}^{23}$  and water are constantly being removed. The problem is to determine the change in total quantity and concentration of the  $\text{Na}^{23}$  (the tracer substance) within the tank for various rates of intake and output of  $\text{Na}^{23}$  (the nontracer substance). Man or any organism or portion of an organism may be compared to the tank see text for details

#### CONSIDERATIONS

1 If **CN**, concentration of the tracer element, is being observed in order to gain qualitative and quantitative information concerning the tracer substance, **N**, and the nontracer substance, **M**, then it is necessary to realize that

(a) **CN** is being affected by the escape of **N** from the tank if **V**, the volume of the compartment, does not change proportionately

(b) **CN** changes if **N<sub>K</sub>** (the excretion of **N**) is zero, but **V** changes

(c) **CN** is dependent upon the relative variations in **N<sub>K</sub>** and **V**. **V**, of course, depends upon the relative variation in **V<sub>I</sub>** and **V<sub>K</sub>**

2 The total quantity of **N**, independent of concentration within the tank at any given time varies with the rate of discharge of **N** from the tank

3 The tank may be considered to be any living organism, such as man, in which a substance is being studied by the tracer technique. Under biologic conditions certain further restrictions would have to be imposed upon the "tank". For example if an electrolyte, whose role in the problem of isotonicity is important, is being traced, a proportionate increase in the volume of water will be necessary whenever the electrolyte accumulates within the tank. This is true to a minor degree when such substances as fat or glycogen are under observation. Therefore if **V**, the volume of the dispersing compartment, varies then the concentration of **N** will vary accordingly. Furthermore, if a

restriction is applied to the tank so that the osmotic force of  $M$  within  $V$  is constant, then the variations in concentration of  $N$  and  $M$  and in the quantity of  $N$ ,  $M$ , and  $V$  will be determined by the relative intake and elimination of  $M$  and  $V$ . *Changes in the concentration and total quantity of  $N$  within the tank will be determined by the relative intake and output of  $M$  and  $V$ .*

*Analogy Between the Tank and Man* — Any living organism as a whole or any part of the organism regardless of size may be compared to the tank. With such analogies however certain factors must be taken into account for example state of equilibrium homogeneity of the compartment and substances under study disturbances in functions concerned with the problem as well as physiologic, physical and chemical peculiarities inherent in the substance being traced and in the compartment in which it resides. These and other matters will reveal themselves as the discussions progress.

In order to simplify the considerations suppose that man is the tank and sodium is the substance  $M$ , under study. Then the sodium compartment  $V$  will consist of all portions of the body throughout which sodium is distributed. The principal sites are extracellular fluids and bone and to a lesser extent cells and proteins. Since the greatest portion of the sodium  $M$ , is found within the extracellular fluids of the body and since the extracellular fluids must remain isotonic to be compatible with life then in man the quantitative relationship of  $M$  and  $V$  is so restricted that accumulation of one within the body is necessarily accompanied by a proportionate accumulation of the other. If a tracer form of sodium  $N$  ( $Na^{22}$ ) is suddenly added to the body of man to trace the ordinary sodium  $M$  then as in the tank absolute and relative variations in  $L$ , the intake of nontracer sodium ( $Na$ ) in  $V_i$  the intake of water, in  $K$ , the output of nontracer sodium ( $Na$ ) and in  $V_o$  the output of water will influence  $CN$  and the total quantity of  $N$  within the body at any time as well as  $N_i$ . If such factors as the rate of intake alone of ordinary sodium ( $Na$ ) will influence the rate of change in concentration or total amount of the tracer sodium ( $Na$ ) then such factors must be evaluated when a substance within the body is being traced since it is necessary to distinguish these influences from those related to metabolic phenomena associated with enzymatic and other similar reactions. For example a decrease in the  $Na$  count in the blood serum may be due to an increase in volume of body water and not to an increased rate of renal excretion of the isotope or an increased rate of excretion of  $Na$  may be due not to a change in sodium metabolic phenomena within the body but to an increased intake in regular sodium within the diet.

For a more thorough understanding of the quantitative and qualitative nature of some important factors independent of purely metabolic processes which influence concentration and total quantity of the tracer substance, certain interesting and essential theorems were devised.

#### MATHEMATICAL CONSIDERATIONS

If we consider the organism as a tank the mathematical theories outlined below will hold, regardless of the complex metabolic processes (physical and

chemical) within the organism. The equations are general and apply to any type of tracer studies within the limitations of the assumptions.

*Symbols —*

$t$  = time

$M_t$  = quantity of *nonttracer* substance in organism at time  $t$

$M_o$  = quantity of *nonttracer* substance in organism initially

$I$  = quantity of *nonttracer* substance taken into organism per unit time

$G$  = net quantity of *nonttracer* substance gained by organism per unit time (negative  $G$  = loss)

$K$  = quantity of *nonttracer* substance eliminated from organism per unit time

$D$  = total amount of *nonttracer* substance obtained under the condition of accumulation

$a$  = fraction of *nonttracer* substance in organism eliminated from organism at any time  $t$  an expression of the rate of elimination

$\alpha$  = fraction of difference between maximum of the *nonttracer* substance to be reached and amount present at time  $t$  an expression of the rate of accumulation

$N_t$  = quantity of *tracer* substance in organism at time  $t$

$N_o$  = quantity of *tracer* substance in organism initially

$V_t$  = volume of compartment under study at time  $t$

$V_o$  = initial volume of compartment under study

$CM$  = concentration of *nonttracer* substance in organism (constant)

$CN_t$  = concentration of *tracer* substance in organism at time  $t$

$CN_o$  = concentration of *tracer* substance in organism initially

*Assumptions*

1. *Tracer* and *nonttracer* substances are uniformly mixed in the organism and are affected similarly by chemical and physical processes in the organism.

2. All the *tracer* substance is added at time  $t = 0$  (initially) and is rapidly mixed completely with the *nonttracer* substance.

3. Intake and discharge are continuous processes.

a. If the quantity of *nonttracer* substance in the organism decreases, the change is expressed by

$$M_t = M_o e^{-at} \quad (1)$$

b. If the quantity of *nonttracer* substance in the organism increases, the change is expressed by

$$M_t = M_o + D(1 - e^{-at}) \quad (2)$$

4. The change of the quantity of the *nonttracer* substance and of the volume of its compartment is assumed to vary exponentially, since this is a physiologic variation which has been observed in our studies.

5. The solution of *nonttracer* substance is always isotonic, therefore, in 3a,

$$V_t = V_o e^{-at}$$

and in 3b,

$$V_t = V_o + D(1 - e^{-at}) = \frac{V_o}{M_o} [M_o + D(1 - e^{-at})] \quad (2a)$$

Three conditions were analyzed

First, what are the variations with time in the concentration and total quantity of the tracer substance when the nontracer substance is in a negative balance, that is, when the rate of output exceeds the rate of intake?

Since the nontracer substance decreases,  $M_t = M_0 e^{-at}$ . In this case

$$G = \frac{dM_t}{dt} = -aM_0 e^{-at} \quad (3)$$

and

$$I = K + G = K - aM_0 e^{-at} \quad (4)$$

or

$$K = I + aM_0 e^{-at} \quad (5)$$

The discharge of the tracer substance from the organism per unit time is

$$\frac{dN_t}{dt} = -K \frac{N_t}{M} = -(I + aM_0 e^{-at}) \frac{N_t}{M_0} = - \left[ \frac{I}{M_0} e^{-at} + a \right] N_t \quad (6)$$

or

$$\frac{dN_t}{dt} + \left[ \frac{I}{M_0} e^{-at} + a \right] N_t = 0 \quad (7)$$

The solution of this first order linear differential equation is

$$N_t = A e^{- \int \left( \frac{I}{M_0} e^{-at} + a \right) dt} = A e^{- \left( \frac{I}{M_0} \frac{1}{a} e^{-at} + at \right)} \quad (8)$$

where  $A$  is the integration constant

Since  $N_t = N_0$  when  $t = 0$ ,  
therefore,

$$N_0 = A e^{- \left( \frac{I}{M_0} \frac{1}{a} + 0 \right)} = A e^{- \frac{I}{aM_0}}$$

or

$$A = N_0 e^{\left( \frac{I}{M_0} \frac{1}{a} \right)} \quad (9)$$

Consequently

$$N_t = N_0 e^{\left( \frac{I}{M_0} \frac{1}{a} \right)} e^{- \left( \frac{I}{M_0} \frac{1}{a} e^{-at} + at \right)} = N_0 \left[ \frac{I}{M_0} \frac{1}{a} (1 - e^{-at}) \right] e^{-at} \quad (10)$$

and

$$CN_t = \frac{N_t}{V_t} = \frac{N_0 e^{\left[ \frac{I}{M_0} \frac{1}{a} (1 - e^{-at}) \right]} e^{-at}}{V_0 e^{-at}} = CN_0 \left[ \frac{I}{M_0} \frac{1}{a} (1 - e^{-at}) \right] \quad (11)$$

*Second*, what are the variations with time in the concentration and in total quantity of the tracer substance when the nontracer substance is in a *positive balance*, that is, when the rate of intake of the substance exceeds the rate of output?

Since nontracer substance increases,  $M_t = M_0 + D(1 - e^{-\alpha t})$  In this case

$$G = \frac{dM_t}{dt} = \alpha D e^{-\alpha t} \quad (12)$$

and

$$I = K + G = K + \alpha D e^{-\alpha t} \quad (13)$$

or

$$K = I - \alpha D e^{-\alpha t} \quad (14)$$

The discharge of the tracer substance from the organism per unit of time is

$$\frac{dN_t}{dt} = -K \frac{N_t}{M_t} = -(I - \alpha D e^{-\alpha t}) \frac{N_t}{M_0 + D(1 - e^{-\alpha t})} \quad (15)$$

or

$$\frac{dN_t}{dt} + \frac{I - \alpha D e^{-\alpha t}}{M_0 + D(1 - e^{-\alpha t})} N_t = 0 \quad (16)$$

The solution of this first order linear differential equation is

$$N_t = A e^{-\int \frac{I - \alpha D e^{-\alpha t}}{M_0 + D(1 - e^{-\alpha t})} dt} \quad (17)$$

where A is the integration constant. The integral may be separated into two parts which are standard forms available in integral tables to give

$$N_t = A e^{-\frac{I}{M_0 + D} t - \left[ \frac{I}{(M_0 + D)\alpha} - 1 \right] \log_e |M_0 + D(1 - e^{-\alpha t})|} \quad (18)$$

Since  $N_t = N_0$  when  $t = 0$ ,  
therefore

$$N_0 = A e^{-\left[ \frac{I}{(M_0 + D)\alpha} - 1 \right] \log_e M_0} \quad (19)$$

or

$$A = N_0 e^{\left[ \frac{I}{(M_0 + D)\alpha} - 1 \right] \log_e M_0} = N_0 M_0^{\left[ \frac{I}{(M_0 + D)\alpha} - 1 \right]} \quad (20)$$

Consequently

$$N = N_0 e^{\left[ \frac{I}{(M+D)\alpha} - 1 \right] \log M - \frac{I}{M+D} t - \left[ \frac{I}{(M+D)\alpha} - 1 \right] \log [M + D (1 - e^{-\alpha t})]} \\ = N_0 M^{\frac{1}{(M+D)\alpha} - 1} [M + D (1 - e^{-\alpha t})]^{-\left[ \frac{I}{(M+D)\alpha} - 1 \right]} e^{-\frac{I}{M+D} t} \quad (21)$$

and

$$CN_t = \frac{N_t}{V_t} = \frac{N_0 M^{\frac{I}{(M+D)\alpha} - 1} [M + D (1 - e^{-\alpha t})]^{-\left[ \frac{I}{(M+D)\alpha} - 1 \right]} e^{-\frac{I}{M+D} t}}{\frac{V}{M} [M + D (1 - e^{-\alpha t})]} \\ = CN_0 M^{\frac{I}{(M+D)\alpha}} [M + D (1 - e^{-\alpha t})]^{-\frac{I}{(M+D)\alpha}} e^{-\frac{I}{M+D} t} \quad (22)$$

*Third*, what are the variations with time in the concentration and total quantity of the tracer substance when the nontracer substance is not changing in amount within the organism that is when intake and output are equal?

Since the nontracer substance does not change  $M_t = M_0$ . In this case

$$\alpha = \frac{dM_t}{dt} = 0 \quad (23)$$

and

$$I = K + G = K \quad (24)$$

The discharge of the tracer substance from the organism per unit of time is

$$\frac{dN_t}{dt} = -K \frac{N_t}{M} = -\frac{I}{M} N_t$$

or

$$\frac{dN_t}{dt} + \frac{I}{M} N_t = 0 \quad (25)$$

The solution of this linear differential equation with the condition  $N_t = N_0$  when  $t = 0$  is

$$N = N_0 e^{-\frac{I}{M} t} \quad (26)$$

Therefore

$$CN_t = \frac{N}{V_t} = \frac{N_0 e^{-\frac{I}{M} t}}{V} = CN_0 e^{-\frac{I}{M} t} \quad (27)$$

## DISCUSSION OF EQUATIONS

So that the significance of the equations may be appreciated, certain theoretic considerations are presented. Because the present studies are concerned with tracing sodium following a single injection of radiosodium ( $\text{Na}^{22}$ ) in normal man and in man with generalized edema due to congestive heart

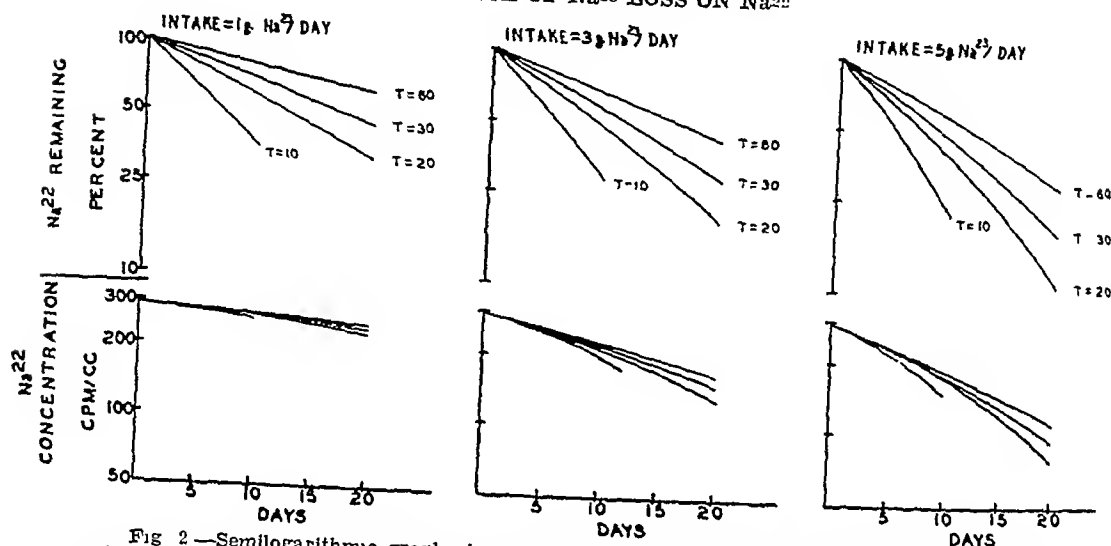
INFLUENCE OF  $\text{Na}^{23}$  LOSS ON  $\text{Na}^{22}$ 

Fig 2 —Semilogarithmic graph showing the influence of the net loss of  $\text{Na}^{23}$  on the  $\text{Na}^{22}$  content of the body after administration of a single dose of the tracer. The influence of the rate of  $\text{Na}^{23}$  intake upon the  $\text{Na}^{22}$  is indicated.  $T$  denotes the time in days required for an edematous individual with a total of 121.4 grams of  $\text{Na}^{23}$  in the body to become edema-free and for the  $\text{Na}^{22}$  content to be reduced to 50 grams. The upper group of curves shows the time variations in the percentage of the initial  $\text{Na}^{22}$  remaining in the body and the lower group of curves indicates the time variations in concentration of the  $\text{Na}^{22}$ . The  $T$  values follow the same order for the lower group of curves as indicated for the upper ones. For the curve showing rapid rate of  $\text{Na}^{23}$  loss or rapid rate of elimination of edema ( $T = 10$  days) the patient reached the edema-free state in ten days and the curve therefore does not extend as far on the abscissa as do those curves for the other rates. It is interesting to note that the curves are *not* straight lines. For convenience the other curves were not continued beyond twenty days if they were extended until the subject became edema-free the amount of  $\text{Na}^{22}$  remaining in the body would be progressively less as the value of  $T$  increased.

failure, it would be interesting and enlightening to apply the equations formulated to certain theoretic situations, such as man with generalized edema who is losing it rapidly, normal man developing edema, and man whose state of electrolyte and water balance is stationary, and to determine the time variations in  $\text{Na}^{22}$  in the body in these three conditions.

*Application 1* —Man with generalized edema such that his total  $\text{Na}^{23}$  mass is 121.4 grams (his total extracellular fluid mass 34,000 grams), his edema is progressively decreasing, and the sodium ( $\text{Na}^{23}$ ) and extracellular fluid masses for a man weighing 70 kilograms (50 and 14,000 Gm respectively) are finally reached.

For better comprehension of the influence of the rate of elimination or discharge of  $\text{Na}^{23}$  upon the concentration and total amount of  $\text{Na}^{22}$  present within the body, the rate with which the subject theoretically became free from edema

was varied for different rates of sodium intake. The output of water was assumed to vary during the defined circumstances to maintain isotonicity.

Figs 2 and 3 show the theoretic progressive change in the total amount and in the concentration of  $\text{Na}^{22}$  in the extracellular fluid for rates of excretion which would make the subject free of edema in ten, twenty, thirty, and sixty days when the daily intake of sodium ( $\text{Na}^3$ ) was 1, 3 or 5 grams.

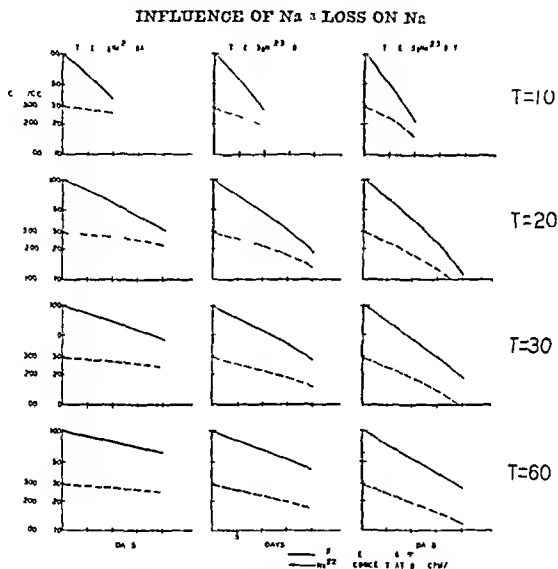


Fig 3—The individual curves shown in Fig 2 grouped to show better the relationship of the time variations of concentration of  $\text{Na}^{22}$  to the time course of the percentage of an initial single quantity of administered  $\text{Na}^{22}$  not eliminated.

These two figures reveal several things

(a) When the intake of  $\text{Na}^3$  remains constant, its rate of elimination so influences the  $\text{Na}^{22}$  in the extracellular fluid that measurements solely of the concentration of  $\text{Na}^{22}$  in the blood serum cannot serve as an index of the amount of  $\text{Na}^{22}$  still remaining in the body. A small decrease in the concentration of  $\text{Na}^{22}$  is associated with a relatively large total elimination of  $\text{Na}^{22}$ . This is to be expected since water is being lost simultaneously at a rate which maintains isotonicity of the  $\text{Na}^{22}$ .

(b) Differences in the rate with which  $\text{Na}^3$  is eliminated from the subject are accompanied by less change in the concentration of the  $\text{Na}^{22}$  in the extracellular fluid than in total quantity of  $\text{Na}^{22}$  present within those fluids. This is reasonable, since water is being eliminated with the  $\text{Na}^3$  to maintain isotonicity.



(c) The rate of  $\text{Na}^{23}$  intake has a greater influence upon the change in concentration of  $\text{Na}^{22}$  in the extracellular fluids than does the rate of elimination of the edema. This is well illustrated by Fig. 3 for the daily intake of 1 and 5 grams. A greater intake of  $\text{Na}^{23}$  results in a more rapid rate of elimination of  $\text{Na}^{22}$  as well as in a sharper rate of decline in concentration of  $\text{Na}^{22}$  in the extracellular fluids. This effect of  $\text{Na}^{23}$  intake upon  $\text{Na}^{22}$  must exist, because the rate of elimination of the former must increase in order to produce the edema.

#### INFLUENCE OF $\text{Na}^{23}$ GAIN ON $\text{Na}^{22}$

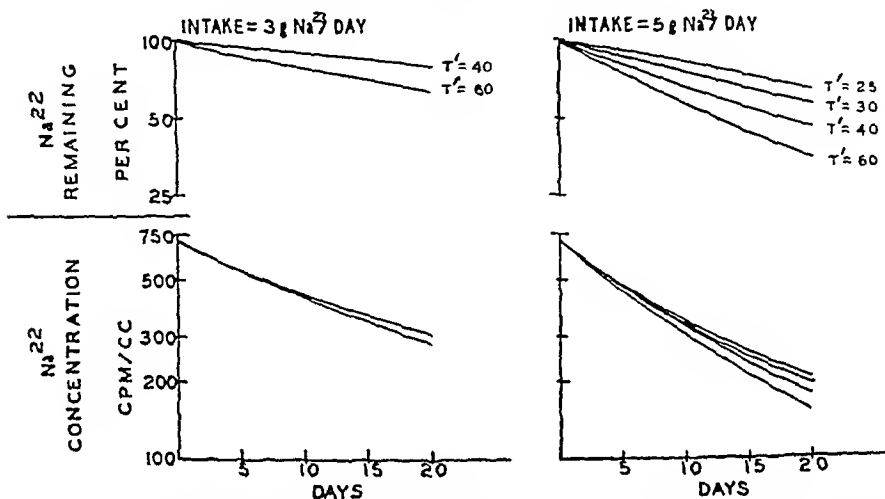


Fig. 4.—Semilogarithmic graph showing the influence of the net gain of  $\text{Na}^{23}$  on the  $\text{Na}^{22}$  content of the body after the administration of a single dose of the tracer. The influence of the rate of  $\text{Na}^{23}$  intake upon the  $\text{Na}^{22}$  is indicated.  $T$  denotes the time in days required for the total sodium of a nonedematous individual to increase from 50 grams to 121.4 grams when an arbitrary maximum of edema is reached. The labeling scheme is the same as used in Fig. 2. Each curve is not a straight line for obvious reasons. Values of  $T$  smaller than those shown could not be analyzed under the conditions of the calculations (see text). Note the inverse relationship in the order on the ordinate positions of the curves for the respective  $T$  and  $T'$  values of this figure and that of Fig. 2.

free state at the given time. Since the movement of the  $\text{Na}^{22}$  is determined by the movement of the  $\text{Na}^{23}$ , then a greater intake of  $\text{Na}^{23}$ , with a resulting increased elimination of  $\text{Na}^{23}$ , must produce an increase in the rate of elimination of  $\text{Na}^{22}$ .

(d) The elimination of  $\text{Na}^{22}$  under the circumstances described is not a simple exponential phenomenon.

(e) It is evident from the equations and from Figs. 2 and 3 that when the subject is losing edema, the rate of excretion of  $\text{Na}^{23}$  is of paramount importance in influencing the  $\text{Na}^{22}$  content of the body, since the volume of the extracellular fluid decreases at a rate which insures isotonicity. The rate of  $\text{Na}^{23}$  intake is influential because of its effect upon its own rate of elimination.

(f) When a subject is progressively excreting the nontracer substance under the conditions defined, the rate of change in the total amount of the tracer substance retained is greater than the rate of change in concentration of the tracer. This is to be expected from equations (10) and (11). It must be remembered

that the volume of the compartment of the tracer and nontracer substances is diminishing progressively as the  $\text{Na}^+$  is being eliminated

(g) The  $\text{Na}^+$  concentration in the extracellular fluids is constant a necessary condition since isotonicity is essential for life.  $\text{Na}^+$  concentration however is not constant, because  $\text{Na}^+$  is gradually being eliminated from the body without any associated continuous addition

### INFLUENCE OF $\text{Na}^{23}$ GAIN ON $\text{Na}^{22}$

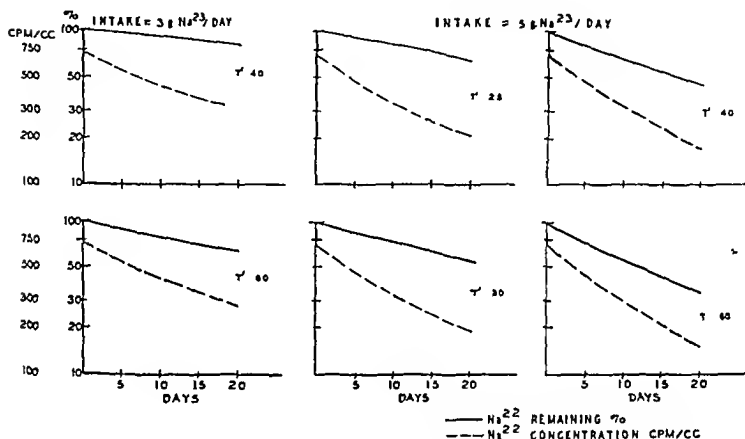


Fig 5—The individual curves shown in Fig 4 grouped to show better the relationship of the time variations of concentration of  $\text{Na}^{22}$  to the time course of the percentage of an initial single quantity of administered  $\text{Na}^{22}$  not eliminated

**Application 2**—An edema free man weighing 70 kilograms whose total  $\text{Na}^+$  mass is 50 grams (his total extracellular fluid mass is 14 000 grams), and in whom edema is progressively developing until a sodium ( $\text{Na}^+$ ) and extracellular fluid masses of 121.4 grams and 34,000 grams respectively are finally reached. Isotonicity is assumed to be maintained

The intakes of  $\text{Na}^{23}$  and water in this man were observed from a theoretic point of view to learn their effects upon the  $\text{Na}^{22}$  concentration in the extracellular fluids and upon the rate of elimination of  $\text{Na}^{22}$ . The influences of the rate of excretion of  $\text{Na}^+$  upon the time variations in concentrations of the  $\text{Na}^{22}$  and upon the rate of elimination of  $\text{Na}^{22}$  in a subject in whom edema is progressively increasing are summarized in Figs 4 and 5. These calculations yield the following information

(a) A subject cannot acquire edema and accumulate sodium at a rate faster than the intake except where special consideration must be given to storage depots and local shifts. For this reason daily intake of 1 gram of  $\text{Na}^+$

could not be studied for the rates of formation of edema considered to exist in these calculations. It was also impossible to study the condition when maximum edema was realized in ten days, as such a limit could not have been reached in this time even when the daily intake of  $\text{Na}^{23}$  was 5 grams. Therefore, the rate with which it is possible for edema to develop is dependent on the rate of intake of sodium.

#### INFLUENCE ON $\text{Na}^{22}$ , AMOUNT OF $\text{Na}^{23}$ CONSTANT

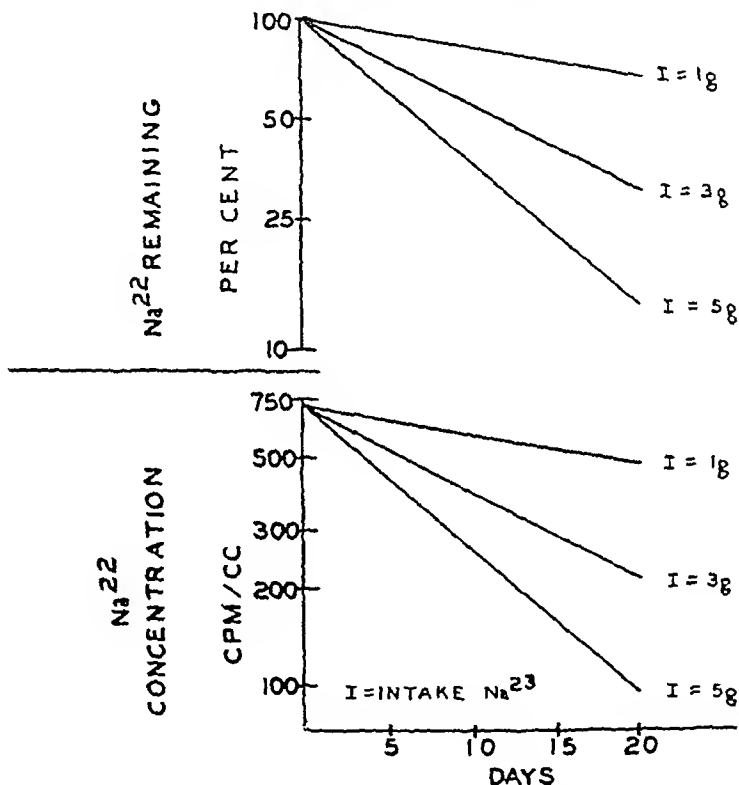


Fig. 6 — Semilogarithmic graph showing the influence of no change in the total  $\text{Na}^{23}$  upon the  $\text{Na}^{22}$  content of the body after administration of a single dose of the tracer. Although the total amount of  $\text{Na}^{23}$  does not change  $\text{Na}^{23}$  is being taken into the body and also being eliminated at rates of 1, 3, and 5 grams daily. These curves are all straight lines for obvious reasons.

(b) When  $\text{Na}^{23}$  is being accumulated during progressive formation of edema, the rate of decrease in concentration of the  $\text{Na}^{22}$  exceeds the rate of change in total content of the tracer within the body. This is to be expected, since the concentration of  $\text{Na}^{22}$  is being reduced by *two* factors acting simultaneously.

(1) Accumulation of fluid of edema, which produces a *dilution effect* on the  $\text{Na}^{22}$ .

(2) *Continuous elimination of  $\text{Na}^{22}$* 

Reduction in the total content of  $\text{Na}$  within the body on the other hand results only from continuous elimination of  $\text{Na}$ .

(c) Intake of  $\text{Na}^{22}$  and its resultant output has a greater influence on the  $\text{Na}$  concentration and total content within the body than the rate of the development of the edema.

*Application 3*—Man whose electrolyte and water balance are stationary.

For the special situation in which there is no gain or loss of  $\text{Na}^{22}$  in the body and in which the extracellular fluid remains isotonic, the rate of elimination of  $\text{Na}^{22}$  becomes all important. The rate of intake is significant because of its direct influence upon excretion. When  $G$  is equal to zero, then intake and output must be equal.

Results of these calculations are shown in Fig. 6, which shows that

(a) The greater the elimination and, therefore, intake of  $\text{Na}^{22}$ , the greater the elimination of  $\text{Na}$ . This follows because the movement of the non-tracer substance determines the movement of the tracer.

(b) When  $G$  is equal to zero, the rates of intake and output have no greater influence on the change in concentration of the tracer,  $\text{Na}^{22}$ , than they have on the change in total quantity of the tracer. This is obvious from equations (26) and (27).

(c) When  $G$  is equal to zero and the tracer substance is not fixed in any manner in the organism, the  $D_1$ ,  $C_{1/2}$ , and  $D_{1/2}$  (time required to eliminate one half of the tracer) are equal.

(d) When  $G$  is equal to zero the decay curves for concentration and for total quantity of the tracer remaining in the organism are parallel and straight lines on semilogarithmic graph paper. These are simple exponential curves.

## GENERAL DISCUSSION

From the preceding discussions and calculations it is obvious that when a tracer is employed to study a substance in an organism, the rate of excretion of the nontracer substance will affect the concentration and quantity of the tracer substance. This is true regardless of changes in size of the nontracer compartment. Factors which influence the elimination of the nontracer must be properly controlled if other physiologic phenomena are to be accurately evaluated. Variations in size of the compartment for the nontracer substance must also be properly controlled and evaluated.

The situations herein discussed were sharply defined and controlled in these calculations, but such is not possible in the average living organism. The intake of the nontracer substance is not uniform at all times; it usually occurs three or four times a day at an irregular rate. Therefore, its rate of elimination is not constant from hour to hour and from day to day. With disease, this irregularity may be even more pronounced. For example, Fig. 7 illustrates the relationship of changes in  $\text{Na}^{22}$  to  $\text{Na}^{23}$  in a man whose sodium is being studied with  $\text{Na}^{22}$  as a tracer. During the first ten days he is free from edema and his intake and output of  $\text{Na}^{22}$  and water are equal. During the next thirty days he retains

$\text{Na}^{23}$  and water, and edema progressively develops, during the following ten days, as his intake and output of  $\text{Na}^{23}$  and water again become equal, his edematous state remains stationary, during the ensuing thirty days his output of  $\text{Na}^{23}$  and water exceed the intake, so that the edema progressively diminishes, and finally he again has an equal intake and output of  $\text{Na}^{23}$  and water while in

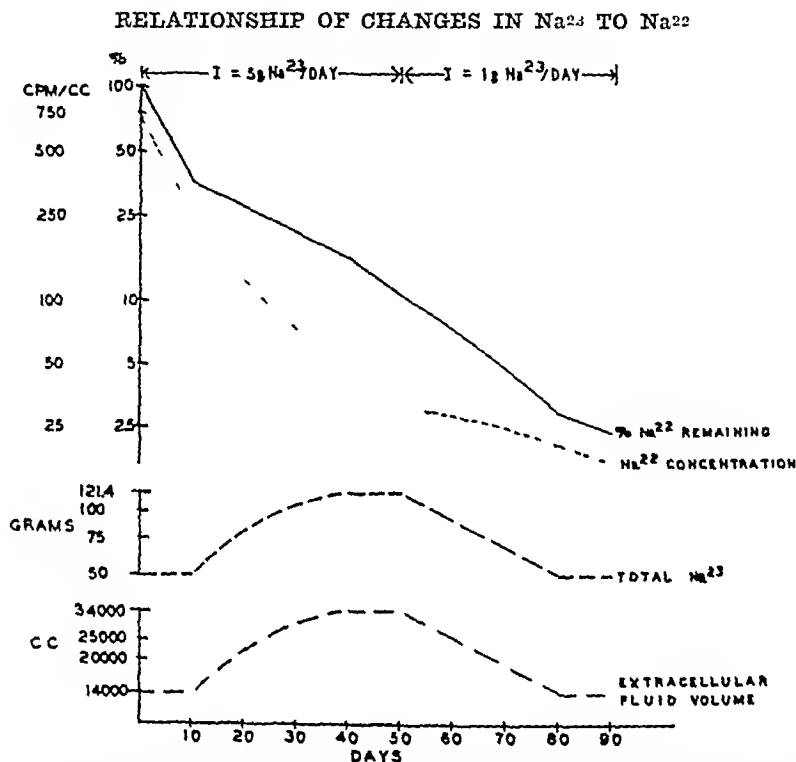


Fig 7—Combination curves illustrating the influence on the concentration of  $\text{Na}^{22}$  and total content of  $\text{Na}^{22}$  in the body for an individual who is normal and edema-free for ten days in whom edema then progressively develops for the next thirty days and who remains at a stationary edematous level for ten days gradually becomes edema-free in thirty days and remains so for ten more days. Consult the text for details

the state free of edema. Curves showing simultaneous variations in concentration and quantity of  $\text{Na}^{22}$  and of  $\text{Na}^{23}$  content within the body are shown. Irregularities in rates of change are attributable to combinations of the three situations previously calculated. Since it is theoretically possible for the time intervals shown on the abscissa to vary from minutes to months, then it is obvious that biologic studies often constitute averages and not detailed absolute values. The conditions are seen to be even more complex when it is realized that all of these and other factors may be acting and varying simultaneously. These data demonstrate the advisability of meticulously controlled biologic experiments.

Complexity of the biologic conditions of experimentation is further evidenced by the fact that absolute equilibrium is relatively rare, if ever present. Furthermore the compartments of the substance under study are not uniform in size, behavior, or constitution, they actually constitute many different compart-

ments within a large one. The behavior of many of the substances traced is under the influence of complex metabolic processes, such as enzymatic reactions further emphasizing the need for great caution in biologic experimentation and in interpretation of data.

### COMPARISON OF THEORETIC WITH EXPERIMENTAL

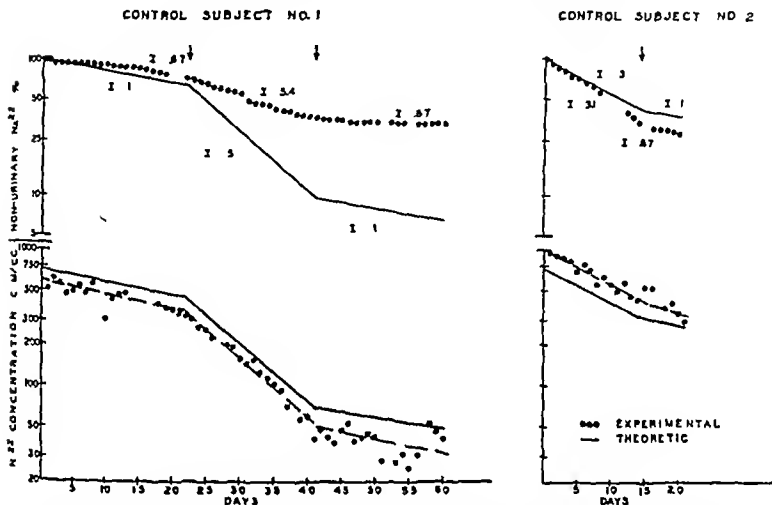


Fig 8—Comparison of the theoretic curves of the concentration of  $\text{Na}^{22}$  and total content of  $\text{Na}^{22}$  in the body of a normal man with data obtained experimentally in two normal subjects. The nonurinary  $\text{Na}^{22}$  is that not excreted in the urine and is represented as percentage of the total  $\text{Na}^{22}$  injected intravenously into the subject at a single dose. For the theoretic curve it represents the percentage of the total  $\text{Na}^{22}$  which is not excreted and remains within the body. Values for intake of  $\text{Na}^{22}$  selected for the theoretic curves were those which approximated the dietary intake in the subjects studied. The theoretic and experimental curves agree remarkably well. Discrepancies between the nonurinary  $\text{Na}^{22}$  curves are due to failure to collect all excretion from the subjects studied experimentally. The experiments were designed for urinary collectors alone.

It is well to emphasize that it is easier to trace elements than complex substances or formed tissue structures, such as cells which enter into metabolic processes and turnover phenomena independent of the element traced. For example, an amino acid tagged with an isotope in which the isotope is followed may lead to difficulties because the isotopic form can be removed from the amino acid molecule and therefore no longer tag the amino acid. The same holds true for cells, as for example, tracing erythrocytes by means of  $\text{P}^{32}$ ,  $\text{Fe}^{59}$  or  $\text{N}^{15}$ . The last of these is the best, since it will remain in the erythrocytes until they disintegrate, whereas the first is least serviceable, since it diffuses out so readily with or without associated metabolic processes.

Obviously, the data herein presented are only theoretic indicating the behavior of the traced substance under the conditions described, and should be

compared with actual biologic experiments only with extreme caution. Nevertheless, it is possible to note a similarity in the curves of the calculations of the theoretic status and those observed with  $\text{Na}^{22}$  in normal subjects and in subjects with congestive heart failure (Fig. 8). That changes in concentration of  $\text{Na}^{22}$  varied with intake of  $\text{Na}^{23}$  and progress of the edema is evident. Unfortunately, the excretion of  $\text{Na}^{22}$  was measured only in the urine and the total  $\text{Na}^{23}$  output was not determined. Such experiments should be conducted.

In the calculations of  $N_t$  the entire loss of  $\text{Na}^{22}$  from an organism was accounted for, therefore when  $N_t$  for  $\text{Na}^{22}$  is equal to one-half of  $N_o$ , then  $t$  is the *overall* or *absolute*  $B_{1/2}$  for the organism. Close inspection of the graphs of the  $N_t$  values reveals variations in the time course of  $N_t$  and therefore  $B_{1/2}$  varies from moment to moment within limiting conditions of the calculations. On the other hand the  $U_{1/2}$  values obtained by experimentation cannot be employed as direct values of  $B_{1/2}$  because the  $U_{1/2}$  values are based only upon urinary excretion of  $\text{Na}^{22}$ .

#### SUMMARY

Equations have been derived which make it possible to predict the influence of variations of intake and output of a nontracer substance and of the size of its compartment upon the concentration within the organism and elimination from the organism of a tracer substance administered in a single dose. These theoretic considerations demonstrate the extreme importance of controlling and properly evaluating factors which affect the rate of elimination and the size of the compartment of the nontracer substance. The need and reasons for exercising every caution in interpretation of data concerned with measuring variations in concentration and total content of the tracer substance are emphasized.

Although the discussion is chiefly concerned with problems for sodium tracer studies, these theoretic considerations apply to any type of tracer experiments, regardless of size or location of the compartment and regardless of whether the tracer is stable or radioactive. It is hoped that the mathematical considerations will be of value to others engaged in biologic and nonbiologic tracer research.

# THE NATURE OF THE ALTERED RENAL FUNCTION IN LOWER NEPHRON NEPHROSIS

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LOWER nephron nephrosis is a term proposed by Luck<sup>1</sup> to describe a syndrome of oliguria with progressive renal insufficiency following a shock like state produced by a variety of acute insults to the body and in many cases associated with the deposition in the renal tubules of various derivatives of hemoglobin and myoglobin. Pathologically, the principal renal lesion is degeneration and necrosis of the epithelium of the ascending loops of Henle and the distal convoluted tubules. We have recently developed a regimen for the management of such patients with lower nephron nephrosis by means of which we were able to keep five out of six patients alive until the kidneys recovered spontaneously and reversed the retention of excretory products. Four patients recovered completely, the fifth still has a residual renal lesion and the sixth died in uremia with heart failure five days after diuresis had set in. The management and the clinical course as well as the detailed serum and urinary chemical findings in these six cases are described elsewhere.

In three of the surviving patients kidney function was studied during the diuretic and recovery periods by means of the specific renal function tests described by Homer Smith and colleagues.<sup>2</sup> Included were serial determinations of the clearances of mannitol, p-aminohippurate, creatinine, and urea and of the tubular excretory mass all performed essentially by the technique described by Goldring and Chasis,<sup>4</sup> except that mannitol was analyzed by the method of Coreoran and Page.<sup>5</sup> The data from these tests obtained during the period of progressive recovery, in addition to the information obtained regarding urinary nitrogen excretion, can in a way be extrapolated back to the period of oliguria and thus provide some insight into the pathologic physiology of the disease.

## RESULTS

The essential clinical features of the six cases are summarized in Table I. It will be seen that the oliguria persisted from five to thirteen days. In the adults it was recognized that once 500 cc of urine were excreted daily, the output thereafter rose rapidly to high levels. The onset of diuresis was accordingly dated from the day 500 cc were first excreted.

Figs. 1, 2, and 3 show the relationship between urinary excretion of water and excretory nitrogen and the serum levels of nonprotein nitrogen in Cases 3, 4, and 5. The tendency for the serum nonprotein nitrogen to level off in spite of the continued intake of protein was either a manifestation of the dilution of retention products produced by the increase in extracellular fluid

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TABLE I CLINICAL DATA ON SIX CASES OF LOWER NEPHRON NEPHROSIS

CASE	PATIENT	SEX AND AGE (YE)	ETIOLOGY	BLOOD PRESSURE		NUMBER OF DAYS OF OLIGURIA	DAY OF MAXIMAL NPN	DAY OF NORMAL NPN	MAXIMAL SERUM NPN (MG/100 CC)
				INITIAL	MAXIMAL				
1	M K	F, 34	Postoperative shock	110/70	186/94	10	11	23	145
2	M B	M, 49	Incompatible transfusion	102/40	180/118	5	10	--	243
3	A V	F, 23	Pneumonia, shock	110/60	148/94	10	14	25	185
4	H K	F, 45	Post partum hemorrhage and shock	58/0	130/80	5	10	22	195
5	L F	M, 52	Carbon tetrachloride poisoning	130/75	150/80	8	11	16	160
6	H P	F, 2	Sulfa reaction, bismuth?	100/60	170/100	13	14	4†	220

\*Death on tenth day

†Serum NPN still 60 mg per 100 c c at 120 days

volume, reflected by gross edema which was deliberately fostered, or of a diminution in cellular break down as the bodily nutrition was improved under the management. It can be seen that the initial days of diuresis were associated with the excretion of relatively small quantities of nitrogen, the concentration ratio of urinary to serum nonprotein nitrogen tending to remain as low as

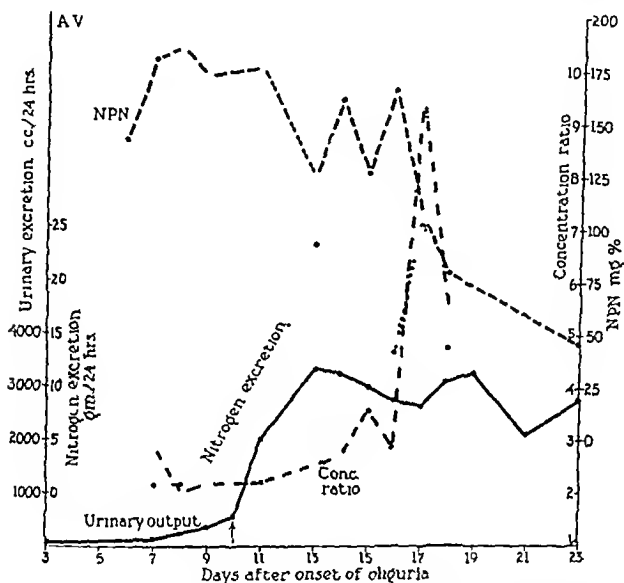


Fig 1—Urinary output of water and nitrogen in Case 3 in relation to serum nonprotein nitrogen concentration and to the ratio of urinary to serum nonprotein nitrogen concentration

during the oliguria period. For this reason, the peak level of serum nonprotein nitrogen was usually not reached until diuresis was well under way. It was only as the diuresis continued to improve (with daily excretions reaching a value of 9,900 cc in Case 5) that the concentration ratio increased. The excretion of nitrogen soon became great enough to produce a rapid fall in the serum nonprotein nitrogen concentration.

Table II shows the absolute values for the various renal functions in Cases 3, 4 and 5. Values for glomerular filtration and effective renal plasma flow were very low at the time of the first test in spite of the fact that these tests were performed when the patients already had improved considerably. Selkurt<sup>6</sup> has demonstrated experimentally that during hemorrhagic shock direct measurements of renal plasma flow give values appreciably higher than those obtained by the determination of *p* aminohippurate clearance. This evidence suggested

that values for mannitol and *p*-aminohippurate clearances were not true measures of the rates of glomerular filtration and renal plasma flow respectively in kidneys as severely damaged as those of our patients. Certain aspects of our data amply confirmed this suspicion. For example, Table II shows that the filtration fraction, as measured by the ratio of the mannitol to the *p* amino hippurate clearance, was in Case 3 greater than 1 instead of the normal 0.20

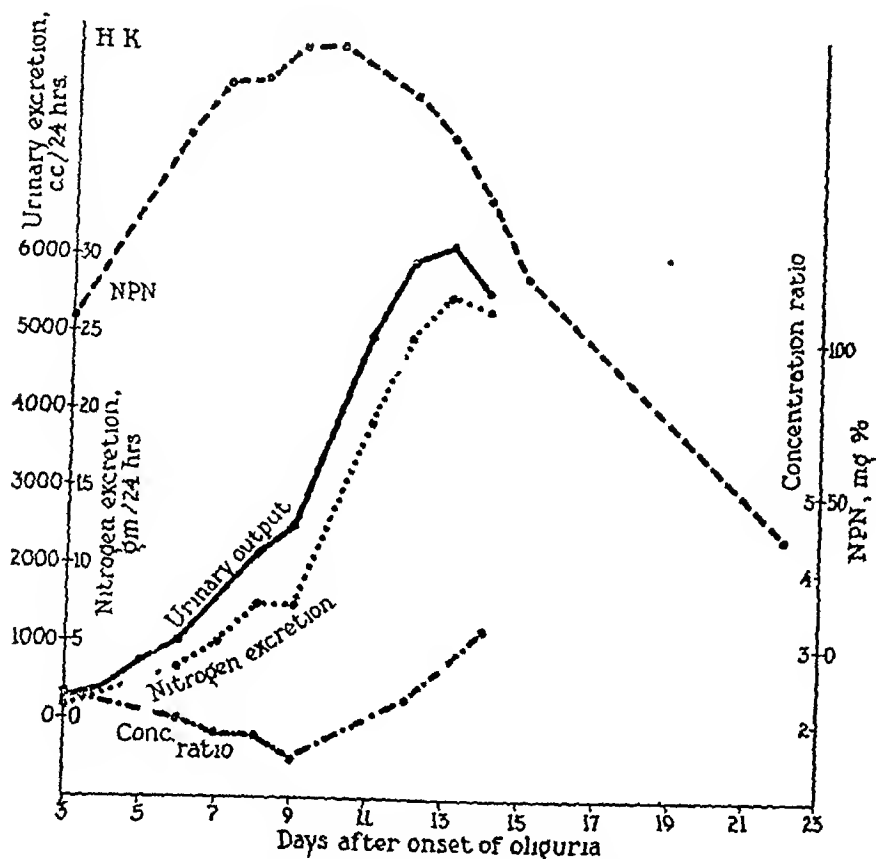


Fig 2—Urinary output of water and nitrogen in Case 4 in relation to serum nonprotein nitrogen concentration and to the ratio of urinary to serum nonprotein nitrogen concentration

Such a finding is, of course, an absurdity. In Case 4, the first filtration fraction was 0.64, a value impossibly high. In Case 5, the first filtration fraction was the normal 0.20, which we believe was due to the fact that the function tests were performed relatively late in a subject who was very rapidly improving.

A further indication of lack of absolute significance of the values in these first tests is the first determination of tubular excretory mass ( $T_m$ ). In Cases 3 and 4 the values were less than zero, which meant that the *p*-aminohippurate excreted by the glomeruli alone—as measured from the mannitol clearance—was greater than the sum of glomerular and tubular excretion as calculated from the total *p* aminohippurate excreted. When this unusual finding was obtained in the first subject it was thought to be due to a technical error. But a second

such result in Case 4 made that explanation unlikely. It was at this time that the paper by Redish and colleagues appeared in which a negative Tm was also reported in their single case. The validity of these analyses was thus further confirmed.

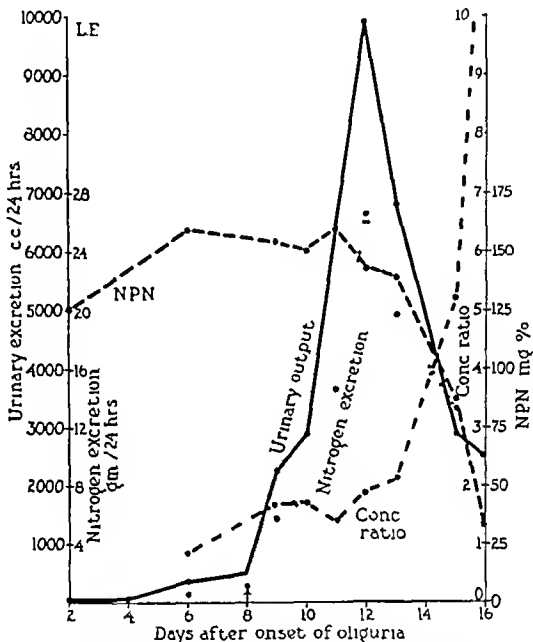


Fig 3—Urinary output of water and nitrogen in Case 3 in relation to serum nonprotein nitrogen concentration and to the ratio of urinary to serum nonprotein nitrogen concentration

Table II also demonstrates still another absurd relationship arising out of the distorted clearance and excretion values during the early stages of recovery. The ratio of the *p* aminoluppurite clearance to the tubular excretory mass which normally lies between 7 and 9 and which is a measure of the relative blood supply to the functioning renal tissue was in Cases 3 and 4, infinitely high because the tubular excretory mass was zero or less than zero. On the other hand, in Case 5 the initial value was only slightly higher than normal.

Fig 4 shows in graphic form the relative rates of restitution of the clearance and tubular mass values toward normal. The distorted relationships found in the first tests disappeared in the subsequent determinations. A week after the first test the tubular mass was no longer negative and the filtration fraction and ratio of *p* aminoluppurite to Tm had returned to nearly normal values in Cases

TABLE II. SIMILAR SPECIFIC RENAL FUNCTION TESTS IN THREE CASES OF LOWER NITRION NEPHROSIS DURING RECOVERY  
(All values have been calculated on the basis of a surface area of 1.73 sq. m.)

CASE	DAYS AFTER ONSET OF OLIGURIA	MANNITOL CLEARANCE (CC/MIN.)	P AMINO HYPPURATE CLEARANCE (CC/MIN.)	P <sub>u</sub> (P AMINOHIP) (MG/MIN.)	UREA CLEARANCE (CC/MIN.)	CREATININE CLEARANCE (CC/MIN.)	FILTRATION FRACTION	CLEARANCE	
								P AMINOHYPPURATE	T <sub>u</sub>
3	17	39.7	38.0	-0.1	16.8	22.9	1.05		∞
	25	55.1	210.0	.69	24.3	40.0	0.26		30.2
	32	55.5	263.0	50.2	63.5	116.3	0.20		5.2
	33	77.3	333.7	---	55.3	---	0.23		---
	191	80.4	732.5	69.1	62.7	109.3	0.11		10.6
4	15	34.7	53.6	-0.1	16	21.2	0.64		∞
	22	51.1	200.7	37.1	32.7	49.6	0.25		5.6
	29	71.3	263.0	53.3	50.3	77.7	0.25		4.9
	56	72.5	420.0	69.4	58.7	106	0.17		6.1
	213	106.6	593.0	83.5	76.5	101.0	0.18		7.1
5	19	51.2	255.0	22.4	31.1	92.4	0.20		11.4
	26	74.1	608.0	54.9	39.9	94.4	0.12		11.1
	33	84.5	544.0	55.7	44.9	114.9	0.16		9.3
	100	126.8	766.0	83.4	73.8	110.6	0.17		9.2
Average normal man		131 ± 21.5	697 ± 136	77.5 ± 13	75 ± 15	120.150	0.20		9.0
Average normal woman		117 ± 15.6	594 ± 102	77.5 ± 13	75 ± 15	120.150	0.20		7.6



nephron to resorb water or sodium and to produce ammonia, thereby forming a concentrated and acidified urine. Instead the tubular cells in this region are so badly damaged that they allow the nonspecific osmotic resorption of most of the modified glomerular filtrate that reaches them, thus leaving little or no filtrate to be excreted as urine. What little urine is excreted during the oliguric phase has a specific gravity not much different from that of glomerular filtrate and is nearly neutral in reaction. The urine obtained during the first days of diuresis is only a larger quantity of the same type of fluid with little or no increase in concentration of the excretory products. It thus appears that the beginning of diuresis is brought about by an increase in glomerular filtration (and effective renal blood flow) without any recovery as yet of specific function of the lower nephron. This increased flow may be brought about by subsidence of renal swelling, as suggested by Coe, Taylor, and Page,<sup>8</sup> or by disappearance of the shunt through nonfiltering glomeruli into the medulla.

The tubular defect is apparently associated with a gradient diffusion of the constituents of the glomerular filtrate. With a high concentration of *p* aminohippurate in the glomerular filtrate and a low concentration in the tubular blood supply (if the excretory function of the proximal convoluted tubules has not been impaired), the *p* aminohippurate is resorbed by the damaged lower nephron to a greater extent than mannitol, the concentration of which is nearly the same in the filtrate as in the tubular blood. As a consequence less *p* aminohippurate may appear in the urine than mannitol in proportion to their respective blood levels. Thus the tubular mass value becomes negative, and the filtration fraction greater than 1. This gradient diffusion appears to be the first tubular defect to recover, as indicated by a return to nearly normal values for filtration fraction and for the ratio of *p*-aminohippurate to tubular mass long before the individual clearances have reached normal. It is reasonable to believe that the improvement in effective renal blood flow is itself responsible for the accelerated tubular repair. The complete recovery of normal renal function in four of our patients and in that of Redish indicates that the glomeruli were never completely blocked and that the tubules always had sufficient blood supply to prevent irreversible destructive changes.

#### SUMMARY AND CONCLUSIONS

Serial determinations of clearances of mannitol, *p* aminohippurate, urea, and creatinine and of tubular excretory mass were carried out in three subjects in the phase of diuresis and recovery from lower nephron nephrosis.

In two subjects, the first test gave negative values for tubular excretory mass and impossibly high values for filtration fraction and ratio of *p* aminohippurate clearance to tubular excretory mass. In subsequent tests these distortions disappeared but complete restitution of renal function did not occur until three to seven months had elapsed.

These data along with the findings on the rate of excretion of nitrogen and water during the oliguric and diuretic periods are consonant with the idea that the renal lesion in lower nephron nephrosis is a diminished renal blood flow in association with a loss of specific function of the lower nephron. Consequently,

the limited amount of modified glomerular filtrate reaching the lower nephron is almost completely resorbed. A gradient diffusion through the damaged cells appears to be present which is responsible for the distorted renal clearance tests. Recovery seems to be produced first by an increase in effective renal blood flow followed later by repair of tubular function. The gradient diffusion is apparently the first tubular defect to disappear. The total recovery of normal tubular function is much slower.

The authors acknowledge with thank the assistance of Mrs. Catherine Noble and Miss Lorraine Schmelzle with the chemical analysis.

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## HISTOPATHOLOGY OF THE LIVER IN HUMAN BRUCELLOSIS

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THE histopathology of brucellosis has been defined for the most part on the basis of studies of the tissues in experimentally infected animals, and the tissues of patients who, having had the disease, died from brucellosis or other causes. Little information is available relative to the tissue reactions that occur in the ambulatory patient with acute or chronic brucellosis. This report represents one aspect of a general study on the pathogenesis of human brucellosis being carried out in this clinic. In these investigations, the changes taking place in certain organs and tissues have been ascertained by obtaining specimens with biopsy techniques. Attention has been given particularly to those patients having bacteriologically proved brucellosis. Sundberg and Spink<sup>1</sup> already have described in detail the histopathology of the sternal bone marrow in human brucellosis. The purpose of this paper is to present the findings in the liver of eleven patients with proved brucellosis. While hepatic lesions have been described previously, there is a lack of knowledge of the changes that occur in living subjects who eventually recover from the disease.

Fabryan,<sup>2</sup> and later Jaffe,<sup>3</sup> emphasized the characteristic granulomatous lesion of brucellosis in experimentally infected guinea pigs. Some years later Löffler and von Albertini<sup>4</sup> described these granulomas in human material. Their initial observations were limited to a single case in which splenectomy and a liver biopsy were carried out. Subsequently, von Albertini and Lieberherr,<sup>5</sup> in one of the best discussions of the pathology of human brucellosis, detailed the findings in the liver and spleen. They observed granulomatous hepatic lesions with and without extensive necrosis. It is quite apparent from studying their paper that brucellosis cannot be distinguished from tuberculosis on the basis of the hepatic changes. The only illustration of a hepatic granulomatous lesion due to brucellosis that we have observed in the English or North American literature is in the paper by Mettler and Kerr.<sup>6</sup> A liver biopsy was secured from the left lobe of the liver at the time a cholecystectomy was performed. Microscopic examination revealed a bizarre, destructive, and inflammatory process in various stages. One large lesion with central necrosis was observed and adjacent to this there were numerous, discrete, tubercle-like foci which consisted of lymphocytes, epithelioid cells, and an occasional giant cell. In another instance Lowbeer<sup>7</sup> reported a case of brucellosis in which cholecystectomy was carried out and a biopsy of the liver was made. Granulomas were found in the wall of the gall bladder and in the liver.

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## METHODS OF STUDY

Specimens of liver were obtained from eleven cases of human brucellosis. The diagnosis of active disease was proved bacteriologically in eight of the eleven cases. In the remaining three patients the diagnosis was established on the basis of epidemiologic, clinical, and serologic evidence. Ten of the eleven patients recovered from the disease. One patient committed suicide and a complete post mortem study was obtained. The duration of the disease in this group varied between three weeks and three years. Biopsies of hepatic tissue in the living subjects were obtained by one of the three following methods: peritoneoscopy, needle biopsy, or surgical excision. The needle biopsy method is now employed exclusively since it is simple and can be carried out at the bed side. In addition to fixing and staining the specimens of liver, portions of the biopsied material in four instances were cultured for Brucella. Special stains were also carried out for connective tissue, reticulum, glycogen, and bacteria.

In an attempt to correlate the histologic changes in the liver with functional impairment of the organ, a series of liver function tests were performed in each case. A brief clinical history of each case is presented along with a description of the hepatic tissue. Pertinent clinical data, the liver function studies that revealed abnormal results and the cardinal histologic findings are summarized in Table I.

## RESULTS

**CASE 1**—H., a 28-year-old housekeeper had been ill for four and one-half months. Weakness was the original complaint. Then ensued severe shaking chills, drenching sweats and fever. The source of her infection was the ingestion of raw milk obtained from a herd of cattle having Bang's disease. On admission to the hospital the outstanding findings were a neutely ill and febrile patient with no demonstrable enlargement of the liver but a firm and nontender spleen was palpated at the left costal margin. Pertinent laboratory data revealed a leukocyte count of 6,400 with a differential of 68 per cent polymorphonuclear neutrophils, 38 per cent lymphocytes, and 4 per cent monocytes. The erythrocyte sedimentation rate was 72 mm in one hour (Westergren). Agglutinins for Brucella were present in the serum in a titer of 1 to 320 to 1 to 640. On four occasions *Brucella abortus* was isolated from cultures of blood.

Penicillin was administered in large doses without benefit but coincident with treatment with sulfadiazine, the patient improved. Subsequent blood cultures, however, showed the presence of *Br. abortus*. Because the patient was febrile and the splenic enlargement persisted, splenectomy was carried out, and a biopsy of the liver was made at the same time. The spleen weighed 217 grams. Multiple cultures of splenic tissue remained sterile, and microscopic studies showed numerous large granulomatous lesions. Subsequent hepatic function studies showed normal values. Splenectomy did not appear to improve her condition, and improvement occurred gradually over a period of several months.

**Study of Liver Biopsy** The specimen was a triangular fragment from the liver edge measuring 9 by 4 by 2 millimeters. Microscopically, the architectural pattern appeared normal. A few of the portal spaces contained a mild infiltration with small round cells. No excess of connective tissue could be demonstrated with the azocarmine stain. Scattered hepatic cells contained a small amount of finely divided golden brown pigment which did not stain for iron. A few degenerating cells were present, surrounded by polymorphonuclear leukocytes. Best's carmine stain showed a normal complement of glycogen. Three small granulomas were present, two located within one portal space and a smaller one within another portal space. They were similar in appearance with a peripheral zone of small lymphocytes and a central collection of epithelioid

TABLE I SUMMARY OF DATA ON ELEVEN CASES OF HUMAN BRUCELLOSIS

CASE	AGE (YR) AND SEX	OCCURRENCE	DURATION OF DISEASE	COMPLICATIONS	BACTERIOLOGY	TITER OF ANTIBODIES	ABNORMAL LIVER FUNCTION STUDIES*	LIVER BIOPSY
1	28 F	Housekeeper	1½ mo	None	Blood + (Br abortus 1 ×)	1 to 320 1 to 640 1 to 2,560	All normal Urino urobilinogen, 2 l Ehrlich units in 2 hr coproporphyrin, 119 g units per 24 hr	Granulomas in portal spaces Granuloma in lobules and portal spaces Langerhans giant cells
2	29 M	Farmer	11 mo	None	Blood + (Br abortus 2 ×)	1 to 5,120	Urino urobilinogen, 36 Ehrlich units in 2 hr urine coproporphyrin, 337 gamma in 24 hr, sulfabromothaleim, 21 per cent retention	Granuloma in lobules and marked infiltration of round cells in portal spaces Patchy fatty metamorphosis
3	17 M	Confectioner	1 mo	None	Blood + (Br abortus 2 ×)	1 to 640	Serum bilirubin 1 mm 0.3 mg, total 1.3 mg	Granuloma with giant cell in lobules, occasional necrotic liver cells
4	33 F	Housewife	22 mo	None	Blood — (repeatedly sterile cultures)	1 to 1,280	<i>First admission</i> Thymol turbidity, 8 units, urino coproporphyrin, 175 gamma in 24 hr <i>Second admission</i> Sulfabromothaleim, 6 per cent retention, cephalin cholesterol flocculation, 2+ in 24 hr, thymol turbidity, 16 units, urino urobilinogen, 52 mg per 24 hr, urino coproporphyrin, 153 gamma per 24 hr	Marked lymphocytic infiltration of liver cells, scattered necrotic liver cells, granulomas in lobules
5	66 F	Housewife	6 mo	None	Blood + (Br abortus 2 ×)			

6	41	M	Truck driver	3 mo	None	Blood + (Br abortus 3 x)	1 to 1.250	All normal	(Granulomas in lobules)
7	61	M	Farmer	1 yr	Left hemiplegia (cause unknown)	Blood + (Br abortus 3 x)	1 to 610	All normal	Moderate fatty metamorphosis with focus of mononuclear cells granulomas
8	56	F	Farmerwife	3 yr	Spondylitis of spine of T <sub>12</sub> with paravertebral abscess <sup>a</sup>	Blood + (Br abortus from blood 2 x)	1 to 1.50	Thymol turbidity 9 units urine urobilinogen 5 units in 1 hr cephalin cholesterol 2+ and 7+ urine coproporphyrin 110 gamma per 24 hr	Granulomas in lobules
9	32	M	Busman	3 wk	None	Blood - (cultures remained sterile)	1 to 5120	All normal	(Granuloma in lobules)
10	25	M	Meat packing plant employee	6 mo	Committed suicide	Blood - (cultures remained sterile)	1 to 120	Thymol turbidity 7 units cephalin cholesterol flocculation 2+ 7+	(Granulomas in lobules and in portal spaces Evidence of healing)
11	30	M	Farmer	5 mo	Noose	Blood - (cultures repeatedly sterile)	1 to 2500	All normal	Moderately severe mononuclear infiltrate of portal spaces Granulomas in lobules

Liver function tests employed routinely included

1 Serum bilirubin (Ducet and Watson J Lab & Clin Med 30 393 1941)

Thymol turbidity (MacLagan Brit J Exper Path 31 34 1941)

3 Cephalin cholesterol (Hanger J Clin Investigation 18 61 1939)

4 Sulfabromthalein (Mateer et al J A M A 111 1313)

5 Quantitative urine urobilinogen (Watson Am J Clin Path 6 48 1936) and/or quantitative urine Ehrlich reaction (Watson et al Am J Clin Path 14 695 1944 and 17 108 1947)

6 Urinary coproporphyrin (Schwartz et al J Biol Chem 163 133 1947)

cells with pink cytoplasm and indented vesicular nuclei as seen in Fig 1. There was one Langhans' type of giant cell with peripherally situated nuclei. Necrosis was absent and reticulum fibers were demonstrated with Wilder's silver stain both peripherally and centrally. No organisms could be demonstrated with Verhoeff's carbofuchsin and MacCallum's modification of Goodpasture's stain.

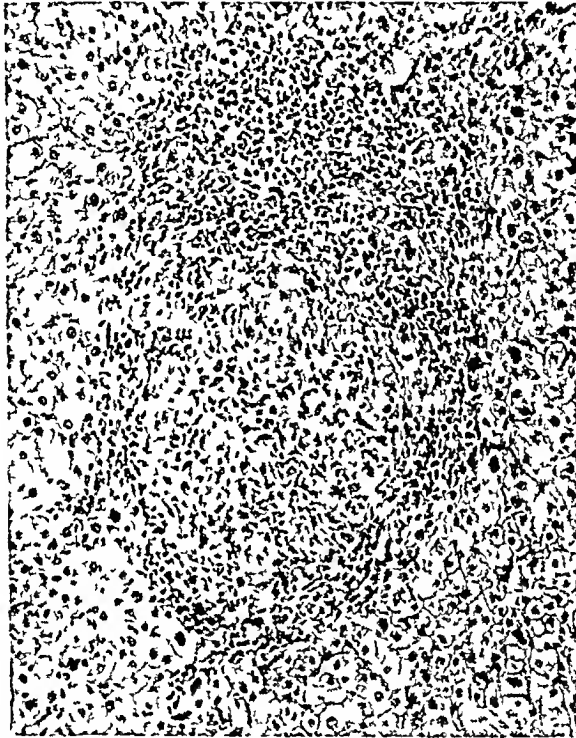


Fig 1.—Photomicrograph ( $\times 175$ ) liver biopsy secured at time of splenectomy in Case 1. The granulomatous lesion consists of a central collection of epithelioid cells and a peripheral zone of small lymphocytes.

CASE 2—L. N., a 29 year old farmer, had been ill over a period of eleven months with weakness, chills, fever and nocturnal sweats. Six months after the onset, a blood culture showed the presence of *Br abortus*, and a course of therapy with sulfadiazine resulted in only temporary improvement. Five months later *Br abortus* was again recovered from a blood culture. Except for enlarged lymph nodes, there were no physical abnormalities. The liver and spleen were not enlarged. Laboratory studies showed a leucocyte count of 6,900 with 56 per cent polymorphonuclear leucocytes, 33 per cent lymphocytes, 8 per cent monocytes, and 3 per cent eosinophiles. The erythrocyte sedimentation rate was 28 mm in one hour (Westergren). The agglutinin titer for Brucella was 1 to 2,560. Liver function studies revealed an excretion of urobilinogen and coproporphyrin in the urine just slightly above the normal range.

Peritoneoscopy was performed. The surfaces of the liver appeared normal, and a biopsy of the liver was secured with the aid of a modified Silverman needle.<sup>3</sup>

**Study of Liver Biopsy.** The biopsy specimens included a slender fragment measuring 10 by  $1\frac{1}{2}$  millimeters. Microscopically there was a normal architectural pattern. Some of the portal spaces contained a few round cells, which

were also present in small numbers in the sinusoids and in focal collections among the hepatic cells. The roentgenogram failed to demonstrate any appreciable increase in connective tissue. The liver cells contained a small amount of finely divided golden brown pigment, part of which stained for iron. In several sections, granulomas with epithelioid cells were noted, the two smallest being in the central portion of a liver lobule and the three larger occupying portal

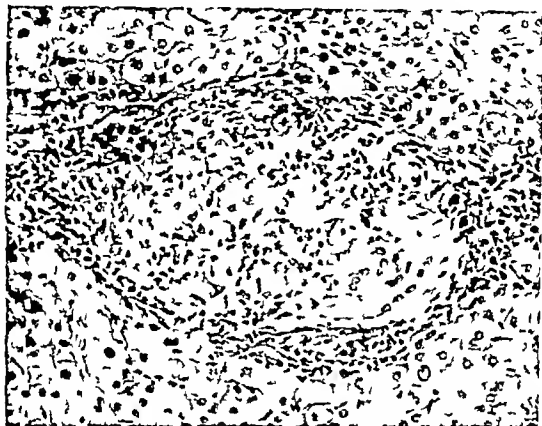


FIG. —Photomicrograph ( $\times 100$ ). Needle biopsy of the liver from Case 2 secured at the time of peritoneo copy. An epithelioid cell granuloma containing Langhans' type giant cells is present in a portal space.

spaces. The smallest lesion consisted of about six epithelioid cells surrounded by approximately a dozen small lymphocytes. The largest occupied the better portion of a small portal space, and showed a peripheral zone of lymphocytes and central epithelioid cell zone as seen in Fig. 2. The cell outlines were indistinct, the cytoplasm having fused, forming syncytial masses resembling Langhans' type giant cells. The nuclei were vesicular, many being indented. Necrosis was absent and reticulum fibers were demonstrated between the epithelioid cells. No organisms were found with acid fast and Gram's stains.

CASE 3—A. M. S., a 47 year old, white, male confectioner became ill one month before entry with fever, drenching night sweats, migratory arthralgia, severe headaches, insomnia, and a marked tremor of his hands. He was a moderately severe alcoholic. His acute illness was contracted through ingesting raw milk and cream obtained from a herd of cattle with Bang's disease. The abnormal physical findings included a tender liver that was felt 4 cm. below the right costal margin. Laboratory data revealed a leucocyte count of 4,100, and a differential count of 49 per cent polymorphonuclear neutrophils, 39 per cent lymphocytes, 5 per cent eosinophiles, 6 per cent monocytes and 1 per cent basophiles. The erythrocyte sedimentation rate was 68 mm. in one hour (Westergren). *Brucella agglutinins* were present in the serum in a titer of 1 to 5120 and *Pr. abortus* was cultured from the blood. Liver function studies showed abnormal excretion of urinary pigments and 21 per cent retention of sulfabromthalein (Table I).

The patient was treated with streptomycin in doses of 0.4 Gm every three hours, but because of the appearance of purpura and an accentuation of the fever, therapy was discontinued. He then received sulfadiazine, and coincident with this improvement in his condition was noted. Although the patient felt better and returned to work, three months after leaving the hospital *B. abortus* was recovered from his blood. After a lapse of another three months, cultures of blood remained sterile.

During the patient's initial stay in the hospital Dr. Richard Vereo secured a biopsy under local anesthesia of the left lobe of the liver through a small midline abdominal incision. Gross inspection and palpation of the small area of the liver that presented itself in the incision revealed no apparent abnormality. A portion of the biopsied specimen was cultured for Brucella; it proved to be sterile.

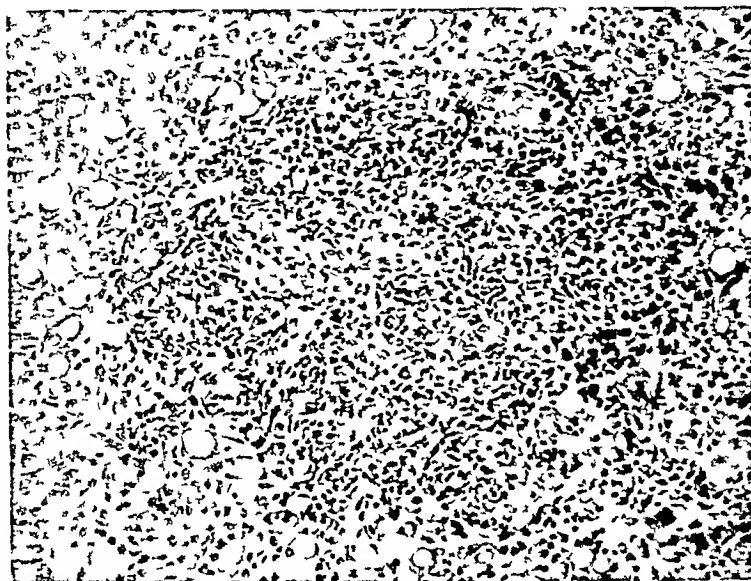


Fig. 3—Photomicrograph (X75) of liver biopsy from Case 3. A confluent granuloma is present as described in the text. In addition there is evidence of a patchy fatty metamorphosis.

*Study of Liver Biopsy* The specimen examined histologically was a fragment from the liver edge measuring 4 by 4 by 3 millimeters. Microscopically, there was a patchy, fatty metamorphosis and a moderately severe, round cell infiltration in the portal spaces. A large epithelioid cell granuloma was present, covering an area slightly greater than a high-power field. Its location with respect to the liver lobule was uncertain, but it did not appear to be within a portal space. It consisted of epithelioid cells, lymphocytes, and a few plasma cells arranged in smaller rounded groups suggesting that it was made up of several smaller confluent granulomas (Fig. 3). In step sections, on which bacterial and other special stains were carried out, the granuloma disappeared and only two small lesions of a similar nature could be found. These consisted of a collection of four or five epithelioid cells surrounded by a dozen or more lymphocytes. No definite bacteria were demonstrated, and there did not appear to be an increase in connective tissue in the portal spaces.

CASE 4—E S, a 55 year old housewife had been ill for twenty two months before she was first seen at the University Hospital. She had experienced marked fatigue for one year before fever and arthralgia appeared. During the time that he was ill a number of cows on her farm were found to have Bang's disease. Physical examination revealed an individual who appeared chronically ill, the complaint of severe headaches and painful joint pain over the lumbar area was aggravated by movement of the legs. The interphalangeal joints of both hands were enlarged and motion was limited. The edge of the liver was just palpable at the costal margin. The leucocyte count was 7500 with 61 per cent polymorphonuclear



Fig 4—Photomicrograph ( $\times 35$ ) of liver biopsy secured in Case 4. An epithelioid cell granuloma containing a giant cell is shown.

neutrophils 35 per cent lymphocytes 3 per cent eosinophiles, and 1 per cent basophiles. The erythrocyte sedimentation rate was 30 mm in one hour. Agglutinins for *Brucella* were present in a titer of 1 to 640. Repeated cultures of blood remained sterile. The only abnormal liver function was a slightly elevated serum bilirubin. She improved while in the hospital but since that time there has been no opportunity to follow her progress.

A biopsy of the liver was obtained through a short midline abdominal incision by Dr K. A. Merendino. The visible surface of the liver appeared normal. A portion of the biopsy cultured for *Brucella* remained sterile.

*Study of Liver Biopsy* A specimen measuring 1 by 5 by 5 cm, from the liver edge was blocked and sectioned. Microscopically the general architecture was normal. Some of the portal spaces, particularly the smaller ones, contained a prominent lymphocyte and plasma cell infiltrate. No increase in fibrous tissue could be demonstrated. Occasional necrotic liver cells were present, surrounded by mononuclear leucocytes as well as a number of the so called regenerative forms with large hyperchromatic nuclei. Many hepatic cells contained a small



... brown pigment part of which stained for iron. Vacuolated cells and small numbers were present around some of the central veins. Approximately ten epithelioid cell granulomas were noted chiefly within lobules, one being in a portal space. Fig. 4 is a photomicrograph of this biopsy. The largest of these granulomas occupied one half the area of a high-power field, the others being much smaller. They consisted of collections of epithelioid cells with vesicular, indented nuclei surrounded by small lymphocytes. Necrosis was absent and reticulum fibers were demonstrated within the lesions. No organisms were seen in preparations appropriately stained.

CASE 5—M. M., a 66-year-old housewife, had pain in the left lower quadrant, in addition to fatigability, night sweats, and weight loss. Her illness had extended over a period of six months. Physical examination revealed a febrile patient having a palpable spleen but the liver was not palpable. The leucocyte count was 3,850 with 51 per cent polymorphonuclear neutrophils, 48 per cent lymphocytes and 1 per cent monocytes. The erythrocyte sedimentation rate was 30 mm. in one hour. The agglutinin titer in her blood for *Brucella* was 1 to 640, and *Br. abortus* was obtained from a culture of blood. Liver function studies revealed an abnormal thymol turbidity test and the urinary coproporphyrin value was elevated.

A peritoneoscopic examination was attempted. The surface of the liver appeared normal, but a satisfactory biopsy was not obtained. Therefore Dr. K. A. Merendino secured a biopsy through a midline abdominal incision under local anesthesia. Part of the specimen was cultured for *Br. abortus* without success.

*Study of Liver Biopsy* The biopsy specimen measured 8 by 4 by 3 mm. and was of normal appearance. Microscopically there was some distortion of the architecture with a suggestion of increased connective tissue and patchy areas of vacuolated liver cells. Many portal areas showed dense lymphocytic infiltration. Throughout there were scattered necrotic liver cells surrounded by mononuclear leucocytes. A few liver cells contained fine brown pigment which did not stain for iron. In step sections, approximately six epithelioid cell granulomas were present at each level. These were of small size consisting of central collections of epithelioid cells varying from five to twenty in number surrounded by a zone of small lymphocytes and occasional plasma cells. A granuloma is shown in Fig. 5. Necrosis was absent and reticulum fibers were demonstrated between the epithelioid cells. No organisms were found in appropriately stained preparations.

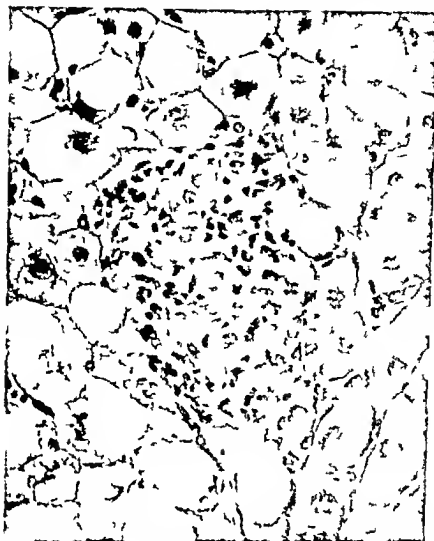


Fig 5—Photomicrograph ( $\times 3$ ) Case 7. One of several granulomas is shown. The surrounding liver cells contain fat vacuoles.



Fig 6—Photomicrograph ( $\times 50$ ) Case 5. The second biopsy was taken six months following the first. Three small granulomas are shown in a single field. The peripheral lymphocytic zone and central epithelioid cells are demonstrated.

liver appeared smooth. A piece of the tissue failed to yield *Brucella* after culturing for the organisms.

The patient was subsequently discharged from the hospital. It was remarkable that the patient experienced so few symptoms in view of the positive bacteriologic studies.

*Study of Second Liver Biopsy* The biopsy specimen measured 10 by 8 by 5 millimeters. Microscopically, the general liver architecture remained unchanged. There was some diminution in the amount of fat present but more necrotic liver cells could be found than in the previous biopsy. In some instances, two to six necrotic liver cells were noted surrounded by mononuclear leucocytes and an occasional polymorphonuclear leucocyte. A transition from these focal necroses to small granulomas was noted. There appeared to be about the same number of granulomas as was noted previously (Fig 6). In one portal space a rounded scar containing small lymphocytes suggested a healed granuloma. Bacterial stains were negative.

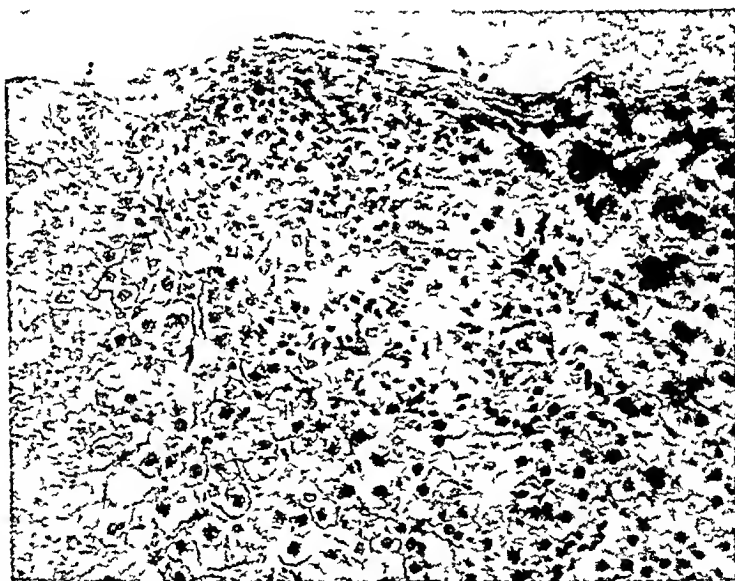


Fig 7—Photomicrograph ( $\times 225$ ). Case 6. One of the granulomas is shown located adjacent to the wall of a central vein.

CASE 6—J. J., a 41-year-old truck driver, had been ill for three months with easy fatigability and lack of energy, succeeded by chills, fever, and night sweats. Four months before the onset of his illness he had worked in a meat packing plant for six months. There were no abnormal findings on physical examination. The laboratory results included a leucocyte count of 4,500 with 40 per cent polymorphonuclear neutrophils and 60 per cent lymphocytes. The erythrocyte sedimentation rate was 77 mm in one hour (Westergren). The agglutinin titer for *Brucella* was 1 to 640, and *Br. abortus* was obtained from a culture of blood. He was treated with sulfadiazine and felt improved. Seven weeks later he was readmitted to the hospital because of chills, fever, malaise, headache, and a nonproductive cough. His temperature was 101.3° F, and the liver and spleen were now palpable. Cultures of blood yielded *Br. abortus*. Liver function studies were within normal limits. A biopsy of

the right lobe of the liver was made with the Silverman needle. He was treated with sulfadiazine again, but because of the appearance of a granulopenia, therapy with this drug was discontinued, and streptomycin was administered. Because of the appearance of a skin eruption, and an accentuation of his fever, treatment with streptomycin was omitted. He finally approached a normal state of health and left the hospital.

*Study of Liver Biopsy* The strip of tissue obtained revealed a fairly normal liver architecture without an increase of connective tissue. The portal spaces contained a moderate mononuclear infiltrate. Scattered liver cells contained fat vacuoles. In all, nine epithelioid cell granulomas were noted which were of varying size, the largest occupying most of a high power field. This is shown in Fig. 7. It was located adjacent to a central vein and consisted of a compact collection of epithelioid cells interspersed with small lymphocytes. Two Langhans type giant cells were present in the center. There was no necrosis. The smallest of the lesions consisted of four or five epithelioid cells forming a rounded mass within a sinusoid.

CASE 7—G. W., a 61 year old farmer, presented a vague history of epigastric distress, recent left hemiplegia, loss of strength, reduction in weight and night sweat. A diagnosis of peptic ulcer had been made in the past. There was no definite evidence of hypertension. In assembling further clinical data it was learned that a herd of cattle owned by the patient had Bang's disease and abortions were occurring. The physical examination was essentially normal except for increased reflexes of the left upper and lower extremities and a liver that was palpable at the costal margin. Laboratory data showed a leucocyte count of 7450 with 50 per cent polymorphonuclear neutrophils, 39 per cent lymphocytes and 11 per cent monocytes. A lumbar puncture revealed normal findings. The erythrocyte sedimentation rate was 29 mm in one hour (Westergren). *Brucella agglutinins* were present in the blood in a titer of 1 to 640, and cultures of blood yielded *Br. abortus*. Liver function studies revealed normal values.

A liver biopsy was carried out with a modified Silverman needle. The patient was treated with a combination of streptomycin and sulfadiazine for two weeks. His response was satisfactory and he left the hospital improved.

*Study of Liver Biopsy* A strip of hepatic tissue showed a mild fatty metamorphosis with frequent focal collections of mononuclear cells and scattered polymorphonuclear leucocytes. Present in these foci were a few epithelioid cells, and in others there were discrete round granulomas composed mainly of epithelioid cells with an occasional Langhans type of giant cell, as illustrated in Fig. 8.

CASE 8—K. S., a 56 year old, white farm wife became ill three years before admission with a bilateral pleuritic pain, fever, night sweats, and pain over the lower part of the spine. In the intervening time she had lost 70 pounds in weight. Two years after the onset she had vaginal bleeding of one month's duration, the cause of which was not ascertained. This was accompanied by a urinary tract infection which responded well to treatment with penicillin. Shortly thereafter, the patient had a cholecystectomy following an acute episode of right upper quadrant colic, vomiting and chills. About nine months later, she was considerably debilitated and was admitted to a tuberculosis sanatorium with a diagnosis of Pott's disease of the spine. Tuberculosis was excluded as a cause of her illness and a diagnosis of active brucellosis with spondylitis was considered. She was admitted to the University Hospitals for treatment. The source of the patient's infection could not be ascertained although she drank raw milk. On examination she appeared poorly nourished and chronically ill. The spleen was palpable 4 cm. below the costal margin, and the liver, 6 cm. below the costal

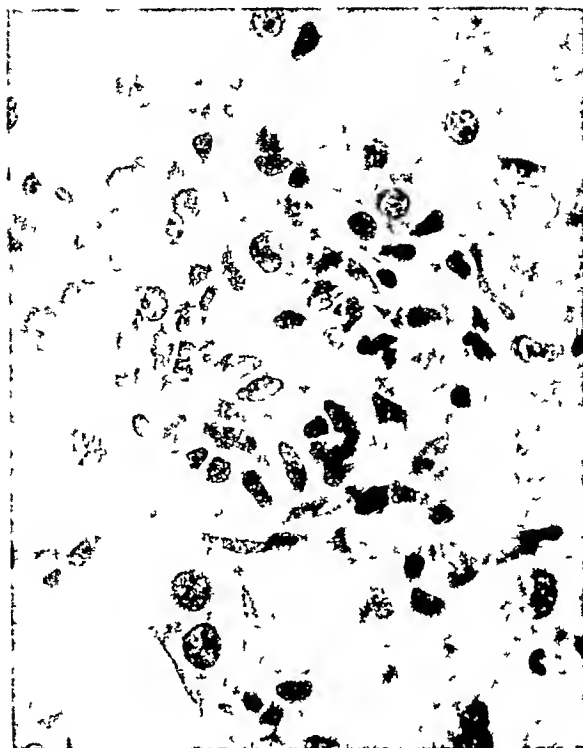


Fig 8—Photomicrograph (X400) Case 7 A small granuloma is present

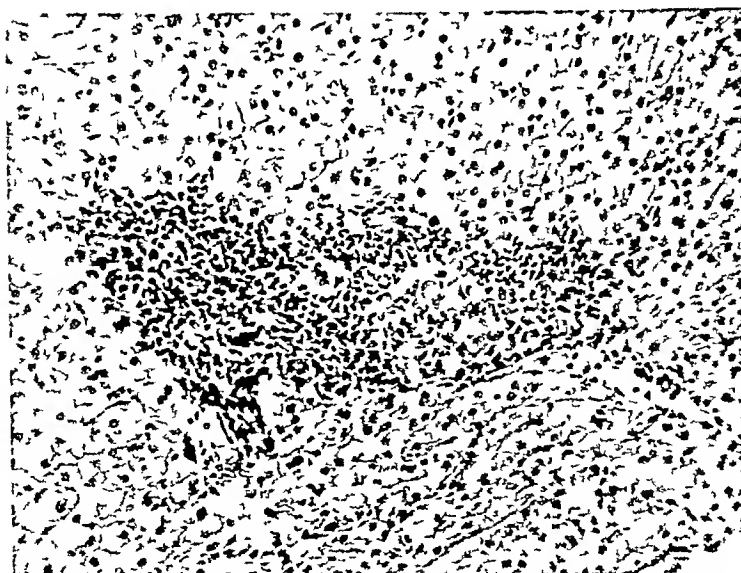


Fig 9—Photomicrograph (X270) Case 8 A small granuloma is present within a portal space.

margin. There was tenderness to percussion extending from the lower thoracic level down to the lumbar area. Laboratory data revealed a leucocyte count of 3,900 with 15 per cent neutrophils, 41 per cent lymphocytes, 7 per cent monocytes, 1 per cent eosinophiles and 1 per cent basophiles. The erythrocyte sedimentation rate was 46 mm in one hour (Westergren). Agglutinins for *Brucella* were present in the blood serum in a titer of 1 to 1:80. *Pr. abortus* was identified in blood cultures on two occasions. Roentgenologic examination of the thoracic spine revealed an inflammatory process involving the seventh and eighth thoracic vertebrae, associated with destruction of the intervertebral disc and a perivertebral mass. Liver function studies showed abnormal findings as given in Table I.

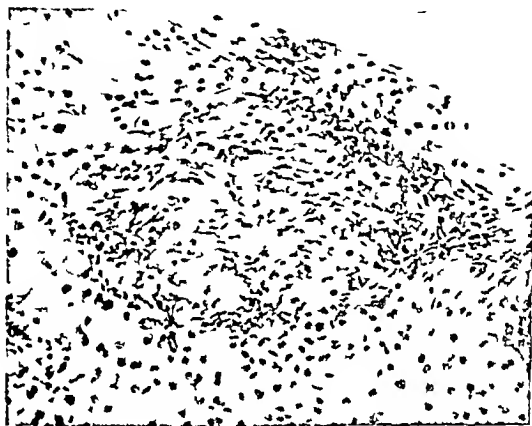


Fig. 10—Photomicrograph ( $\times 400$ ) Case 9. A small granuloma of moderate size is shown.

A liver biopsy was carried out at the bedside with a modified Silverman needle. The patient was treated with a combination of streptomycin and sulfadiazine, receiving 0.5 Gm of the former every six hours for two weeks, and 1 Gm of sulfadiazine every four hours for a similar period. She improved remarkably in this period, and left the hospital. A checkup one month later showed her to be afebrile and abacteremic, and feeling greatly improved.

**Study of Liver Biopsy.** The specimen measured 2 cm in length and 1 mm in diameter. There was an extensive mononuclear infiltrate of the portal areas, but there was no increase in connective tissue. Scattered throughout both portal areas and within liver lobules were granulomas of varying size, the largest of these approximating the measurements of a portal area. Fig. 9 shows a small granuloma within a portal space. The lesions were formed by epithelioid cells surrounded by varying numbers of lymphocytes. Central necrosis was absent. The liver cells contained some fine brown pigment and scattered regenerative and degenerative forms were present.

**CASE 9**—A 32-year-old businessman had the onset of fever and sweats about three weeks before entry to the hospital. Sulfonamide and intror penicillin were given without coincident improvement. There were no abnormal findings on physical examination. Data from laboratory procedures showed a leucocyte count of 8,300 with 56 per cent polymorphonuclear

neutrophils, 30 per cent lymphocytes, and 7 per cent eosinophils. The erythrocyte sedimentation rate was 44 mm in one hour (Westergren). Agglutinins for *Brucella* were present in a titer of 1 to 5,120, but repeated cultures of blood remained sterile. Liver function studies showed normal values.

A biopsy of the liver was performed with a modified Silverman needle. A portion of the tissue was cultured unsuccessfully for *Brucella*. The patient was treated with a combination of streptomycin and sulfadiazine over a period of two weeks. He felt considerably improved on discharge from the hospital. One month later he was afebrile and had a negative blood culture.



Fig. 11—Photomicrograph ( $\times 150$ ) Case 10 showing a large granuloma in liver studied post mortem

**Study of Liver Biopsy** A strip of liver showed a moderate fatty metamorphosis. There was a heavy lymphocytic infiltrate in the portal areas. Well-formed epithelioid tubercles were present, one of which filled almost the entire high-power field in the portal space. This lesion consisted of a central collection of epithelioid cells and a peripheral zone comprising lymphocytes and plasma cells without necrosis. Fig. 10 shows a granuloma.

**CASE 10**—R. B., a 25-year-old meat packing plant employee, had been ill for six months complaining of chills, sensations, fever, and night sweats. He was quite nervous. On examination the outstanding feature was a firm spleen palpated 6 cm below the costal margin. Laboratory information included a leucocyte count of 4,700 with 43 per cent polymorphonuclear neutrophils, and 53 per cent lymphocytes. The sedimentation rate of the erythrocytes was 66 mm in one hour (Westergren). Agglutinins for *Brucella* were present in the blood in a titer of 1 to 5,120. Blood cultures remained sterile. Liver function studies performed five months before the liver was studied histologically revealed abnormal values as shown in Table I.

The patient was not treated specifically, but he abstained from work and rested. He appeared to be getting along fairly well, when he took his life by hanging. A complete post mortem study was carried out by Dr. Robert Hebbel and the only significant histologic findings related to the liver.

*Study of Liver (Post Mortem)* The liver weighed 1 850 grams. Its capsule was smooth. The markings on sectioning the organ were normal. The gall bladder contained greenish bile. The wall appeared normal and the extrahepatic bile ducts were patent and unchained. Microscopic examination showed the presence of scattered granulomas in the liver. The largest occupied the greater part of a high power field. They were round to oval, located within portal areas and within liver lobules. Fig. 11 shows a granuloma beneath the capsule.

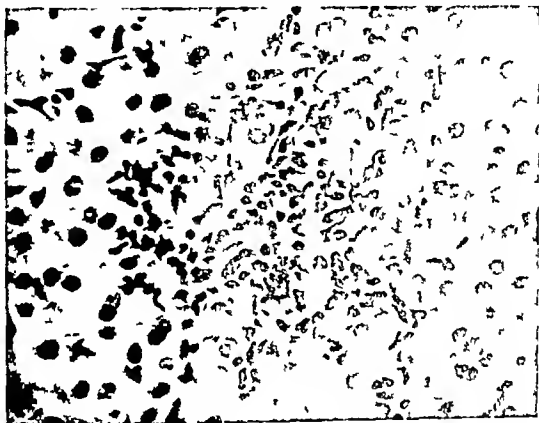


Fig. 11.—Photomicrograph ( $\times 100$ ). Case 11 showing a small granuloma.

There was a predominance of small lymphocytes and plasma cells with a lesser number of epithelioid cells grouped centrally. No giant cells were noted and necrosis was absent. The smaller lesions consisted of dense collections of lymphocytes and plasma cells. It is of interest that several of these larger foci showed evidence of healing consisting of a diminution of cellularity with an increase in collagen fibers.

CASE 11.—H. S., a 35-year-old white farmer had been ill for five months. The onset was marked by severe chills, fever, anorexia, and weakness. During the course of his illness he had lost 49 pounds in weight. Night sweats had been prominent. The patient was severely exposed to Bang's disease existing in a herd of cattle. He had removed and retained placenta with his unprotected hands. He had also ingested raw milk from this herd. It is of interest that three years previously he had had hepatitis of unknown etiology. There were no physical abnormalities apparent on physical examination except for generalized lymphadenopathy and a skin eruption consistent with erythema multiforme. Laboratory data showed a leucocyte count of 6 000 with 52 per cent polymorphonuclear neutrophils, 39 per cent lymphocytes, 4 per cent eosinophiles, 4 per cent monocytes, and 1 per cent basophiles. *Brucella* agglutinins were present in his blood in a titer of 1 to 640. Repeated cultures of venous blood remained sterile. Liver function studies were all within normal limits.

A liver biopsy was obtained at the time of peritoneoscopy. The surfaces of the liver appeared normal. The patient was treated with streptomycin and sulfadiazine for a period of two weeks. He improved markedly and has felt well since leaving the hospital.



*Study of Liver Biopsy* There were two thin fragments of liver measuring 1 and 2 cm in length and 1 mm in diameter. The portal area showed a moderately severe infiltrate with mononuclear cells. Fig. 12 shows a small granuloma. Within several liver lobules there were small granulomas consisting of epithelioid cells and a surrounding zone of lymphocytes.

#### DISCUSSION

An outstanding feature in this study of eleven patients is that hepatic lesions have been present in every instance. The presence of granulomas in the liver lobule or in the portal areas, with a portal cellular infiltrate in several cases indicates that these hepatic lesions cannot be considered as complications but as a part of the natural course of the disease. The presence of the hepatic lesion has no relationship to the severity of the infection or to the clinical status of the patient. The changes have been observed in individuals who were quite ill, and in others who were symptomatically well at the time the biopsy was made.

The genesis of these lesions is worthy of comment. Thus far, in the human investigations, the hepatic changes have been associated only with *Br. abortus*. This species is usually less invasive than *Brucella suis* or *Brucella melitensis*. The appearance of epithelioid cells in the liver lobules, and in the portal spaces, undoubtedly represents a response to the invasion of cells by *Br. abortus*. This infiltration with epithelioid cells indicates either an efficient defense mechanism or invasion of the tissue by microorganisms of low virulence. From observations being conducted in this laboratory by Dr. A. I. Braude in experimentally infected animals,<sup>9</sup> it appears that a combination of these factors is responsible for the noncaseating granuloma. He has observed severe caseation occurring with *Br. suis* and *Br. melitensis*. He has also observed that just a few days before the appearance of epithelioid cells there is an intracytoplasmic invasion of the hepatic cells by demonstrable *Brucella*, but without any surrounding cellular reaction. In the present investigations, attempts were made to culture a portion of the hepatic tissue containing granulomas obtained from four patients, and in each instance the cultures remained sterile.

The hepatic lesions that have been described in this report are not specific for brucellosis. The granulomas in the bone marrow of patients having brucellosis could not be distinguished from sarcoidosis, as pointed out elsewhere.<sup>1</sup> This also applies to the hepatic granulomas and sarcoidosis. Obviously, the histologic diagnosis of involvement of the liver in sarcoidosis should not be made until brucellosis has been carefully ruled out. In addition, the granuloma of brucellosis cannot be differentiated from that observed in some cases of tuberculosis and syphilis.

The simultaneous performance of a battery of liver function tests and an hepatic biopsy has shown little or no hepatic dysfunction in several of the patients having demonstrable changes in the tissues. Instances of hepatitis, a diagnosis based upon clinical and biochemical evidence of liver dysfunction, have been described in patients with brucellosis.<sup>10, 11</sup> A question which this study has provoked is whether brucellosis will cause extensive and permanent

hepatic changes such as cirrhosis of the liver. This aspect of the problem will be considered in another paper. Individual instances of cirrhosis occurring in brucellosis have been reported in this country.<sup>1, 13, 14</sup> Frequent reference to this association has been made from time to time in the continental literature.<sup>1, 4</sup> In this laboratory, it has not been possible thus far to produce cirrhosis of the liver in experimentally infected animals. While a direct relationship between hepatic cirrhosis and human brucellosis has not been clearly established there is accumulating evidence that brucellosis may be a major accessory agent in the genesis of severe and, at times, fatal cirrhosis.

The accurate diagnosis of brucellosis is dependent upon laboratory tests including cultural and serologic methods. Too frequently bacteriologic evidence for brucellosis is lacking, and the remaining data may be inconclusive, or even conflicting. Therefore any procedure that will aid in the diagnosis of active brucellosis will be looked upon favorably by clinicians. The present studies indicate that demonstrable histologic changes may be present in the livers of at least the majority of patients having active brucellosis. The removal of an adequate specimen of liver by means of a needle biopsy may be a useful diagnostic procedure in the more doubtful cases. Although more satisfactory treatment is now available for brucellosis in the form of combined treatment with streptomycin and sulfadiazine one does not like to submit doubtful cases to such therapy because of the expense entailed and also because of the potential toxic reactions either of the two drugs may induce.

#### SUMMARY

1 Biopsies of the liver have been carried out in ten patients with active brucellosis. The histologic appearance of tissue obtained post mortem has been described for an eleventh case. A battery of liver function tests was carried out simultaneously with the biopsies in ten cases.

2 The basic hepatic lesions are granulomas in the lobules and portal spaces along with a cellular infiltrate in the portal areas. The granuloma is not specific for brucellosis and cannot be distinguished from sarcoidosis, and, in some instances from the lesions of tuberculosis and syphilis.

3 In several instances there was little or no deviation from normal in the liver function tests, although definite histologic changes were apparent. While a direct relationship between brucellosis and cirrhosis of the liver has not been established, there is accumulating evidence that brucellosis may play an important accessory role in the genesis of cirrhosis.

4 It is suggested that biopsy of the liver may be employed for diagnostic purposes in doubtful cases of human brucellosis.

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# THE TREATMENT OF PNEUMOCOCCAL PNEUMONIA BY PENICILLIN IN AQUEOUS SOLUTION AT LONG INTERVALS

EFFECTIVENESS OF TWO DOSES IN FIRST TWENTY-FOUR HOURS FOLLOWED BY  
SINGLE DAILY INJECTIONS

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THE technique of administering penicillin has been the subject of considerable investigation since the original discovery that the tubules of the kidney eliminate penicillin rapidly from the blood. Thus many attempts have been made to develop preparations which would either permit penicillin to be absorbed slowly from a depot into which it was injected or which would retard its excretion from the body.<sup>1-3</sup> These efforts were based upon the assumption that constant "therapeutic" blood levels are necessary for the successful treatment of most infections. In the early experience of Tillet,<sup>4</sup> however, and also more recently, evidence has been presented which casts doubt upon the necessity for continuous blood levels in all situations.<sup>5-10</sup>

An opportunity to subject these considerations to clinical trial existed in the Cincinnati General Hospital, where many patients with pneumococcal pneumonia are treated and where typing of pneumococci is still carried out. During the winter of 1947-1948 therefore the efficacy of one or two daily doses of crystalline penicillin G in distilled water was investigated in a series of 118 cases of pneumonia, sixty-four of which were caused by demonstrated typed pneumococci. It is the purpose of this communication to describe the results of this regimen, which has been associated with a lower mortality rate for pneumonia than has ever been observed in this hospital.

*Plan of study*—Beginning Dec. 1, 1947, all patients admitted to the Medical Service of the Cincinnati General Hospital with a primary diagnosis of pneumonia were treated with penicillin but no other chemotherapeutic agents. The only exceptions were cases where meningitis or endocarditis complicated pneumonia and where the pneumonia was believed to be caused by gram-negative bacilli. Streptomycin and sulfonamides were administered in addition to penicillin when gram-negative bacilli were believed to be the cause of the pneumonia; such cases are not included in the present series.

Before penicillin was administered, a sample of sputum was cultured in blood broth for typing of pneumococci, and a blood culture was made. The latter consisted of 3 cc. of blood added to 50 cc. infusion broth in a flask, and an agar pour plate prepared from 1 cc. of the patient's blood. An x-ray of the chest was made in every case within twenty-four hours of admission, and follow-up films were made in most cases.

Three dosage schedules were investigated (Table I). At the beginning of the study patients admitted on the odd days of the month, i.e. the 1st, 3rd, 5th, etc. were given 300,000 units of crystalline penicillin G in 3 cc. distilled water intramuscularly as soon as the diagnosis of pneumonia was made and the material for blood and sputum cultures secured. This

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TABLE I INTRAMUSCULAR PENICILLIN DOSAGE SCHEDULES INVESTIGATED IN THIS STUDY

NAME OF SCHEDULE	DOSAGE	WHEN USED
300,000 series	300,000 units crystalline penicillin G in 3 c.c. distilled water on admission, and every 24 hours for 6 days or until temperature is normal for 48 hours, 1 "booster" dose" given 12 hours after first dose only	Odd days of month, Dec 15, 1947, to June 1, 1948
200,000 series	Same as 300,000 series, except that each dose consisted of 200,000 units in 2 c.c. distilled water	Even days of month, Feb 21, 1948, to June 1, 1948
25,000 q 3 h series	25,000 units crystalline penicillin G in saline or distilled water every 3 hours for 6 days or until temperature was normal for 48 hours	Even days of months, Dec 15, 1947, to Feb 20, 1948

dose was repeated every twenty four hours for six days or until the temperature was normal for forty eight hours. In addition to the daily dose, a "booster" dose of 300,000 units in distilled water was given twelve hours after the initial dose, *on the first day only*. This schedule, which will be referred to as the "300,000 series," was continued throughout the entire period of the investigation, from Dec 15, 1947, to June 1, 1948.

Patients admitted on the even days of the month were given penicillin according to the accepted conventional method, i.e., 25,000 units in saline or distilled water every three hours, for six days or until the temperature was normal for forty eight hours. This schedule was adhered to from Dec 15, 1947, until Feb 20, 1948. At that time it was apparent that clinical results with the two schedules were indistinguishable. It seemed advisable to determine, therefore, whether 200,000 units might be as efficacious as 300,000 as the basic dosage in the "one a day" treatment. The 25,000 units every three hours program was, therefore, terminated, and in its place was substituted a schedule identical with that employed on the odd days of the month, except that 200,000 units crystalline penicillin G in 2 c.c. distilled water were used instead of 300,000 units. This schedule, which will be referred to as the "200,000 series," was followed from Feb 20, 1948, through June 1, 1948.

#### ANALYSIS OF THE CASE MATERIAL

*Distribution According to Type*—Pneumococci were recovered from the sputum of sixty-four of the 118 cases in the 200,000 and 300,000 series. Tabulation of the type distribution (Table II) showed that the commonest types were, in order 2, 7, 4, 3, 14, 1, 5, 9, and 18.

The cases from which no pneumococci were recovered included an assortment of pulmonary conditions, and have not been analyzed in detail. Most of them presented the clinical picture of pneumococcal pneumonia, some more closely resembled "virus pneumonia", and a few were cases of acute infections superimposed upon chronic lung disease such as bronchiectasis. Though they cannot properly be included in mortality statistics for pneumococcal pneumonia, they are worth presenting at this time because the patients were all acutely and at least moderately ill, as patients must be to gain entrance to the wards of the Cincinnati General Hospital.

*Bacteremia*—Twenty of the sixty-four pneumococcal cases had positive blood cultures, an incidence of 31.2 per cent. The distribution of bacteremia among the 200,000 and 300,000 series is presented in Table III.

TABLE II DISTRIBUTION OF CASES ACCORDING TO TYPE OF PNEUMOCOCCUS

	100,000 SERIES	200,000 SERIES	TOTAL	PER CENT TOTAL
Pneumonia diagnosed but no pneumococci recovered	20	54	74	115
Typed pneumococci recovered	18	46	64	
Type distribution				
1	0	2		
2	1	12		
3	1			
4	1	4		
5	0	1		
6	0	1		
7	6	1		
8	0	2		
9	0	1		
10	0	1		
11	0	4		
12	1	0		
13	1	2		
14	0	1		
15	1	1		
16	1	1		
17	2	0		
18	1	0		
19	1	1		
20	1	1		
Total	18	46		

TABLE III INCIDENCE OF BACTERAEMIA IN PNEUMOCOCCAL CASES

	NUMBER OF CASES (NONBACTERAEMIC PLUS BACTERAEMIC)	PER CENT BACTERAEMIC
100,000 series	18	39.0
200,000 series	46	28.2
Total	64	31.2

*Extent of Pneumonia*—Table IV shows that though the pneumonia in most of the cases was restricted to a single lobe portions of two or more lobes were involved in 32 per cent. The higher incidence of multilobar pneumonia in the 200,000 than in the 100,000 unit series was due to chance.

TABLE IV DISTRIBUTION OF PNEUMOCOCCAL CASES ACCORDING TO NUMBER OF LOBES INVOLVED

	1	2	3	4	5
100,000 series	17	1			
200,000 series	20	17	1	1	1
Total	37	18	1	1	1

Portion of a lobe counted as one lobe

*Age of Pneumonia at Onset of Treatment*—The effect of campaigns to convince the public of the importance of early treatment of disease is borne out by the fact that 64 per cent of the patients in this series were admitted on or before the fourth day of their pneumonia (Table V). This is a high proportion for a city hospital as compared with the experience ten or fifteen years ago.

*Distribution of Cases According to Age of Patients*—Table VI presents the age distribution of the cases by decades. The patients were approximately evenly divided between those 40 and under and those over 40.

TABLE V DISTRIBUTION OF PNEUMOCOCCAL CASES ACCORDING TO DURATION OF PNEUMONIA WHEN TREATMENT WAS BEGUN

	DAYS								MORE THAN 7	UNKNOWN
	1	2	3	4	5	6	7			
200,000 series	2	3	6	1	1	0	2	2	1	
300,000 series	5	7	10	7	5	3	3	6	0	
Total	7	10	16	8	6	3	5	8	1	

TABLE VI DISTRIBUTION OF PNEUMOCOCCAL CASES ACCORDING TO AGE OF PATIENT

	13 20	21 30	31 40	41 50	51 60	61 70	71 80	81 90	TOTAL	% 40 AND UNDER	% OVER 40
200 000 series	2	3	6	4	0	1	2	0	18	55.6	44.4
300,000 series	4	11	7	4	4	10	5	1	46	47.8	52.2
Total	6	14	13	8	4	11	7	1	64	51.5	48.5

## RESULTS OF TREATMENT

The results of treatment in the sixty-four pneumococcal cases are summarized in Table VII. There were four deaths, a mortality of 6.3 per cent. When the bacteremic cases were calculated separately from the nonbacteremic, we found a mortality rate of 15 per cent for the former and 2.3 per cent for the latter. In the small series of cases treated with 200,000 units at a time, the mortality was almost the same as in the 300,000 series.

TABLE VII MORTALITY IN SIXTY-FOUR CASES OF PNEUMOCOCCAL PNEUMONIA

	NONBACTEREMIC CASES			BACTEREMIC CASES			BACTEREMIC AND NON BACTEREMIC CASES		
	NUM BER	DEATHS	% MOR TALITY	NUM BER	DEATHS	% MOR TALITY	NUM BER	DEATHS	% MOR TALITY
200,000 series	11	0	0	7	1	14.3	18	1	5.5
300,000 series	3	1	33.3	13	2	15.4	46	3	6.5
Total	44	1	2.3	20	3	15.0	64	4	6.3

The clinical response with both dosage schedules was rapid in most cases and dramatic in some. Often by twelve and usually by the end of twenty-four hours after the first dose the temperature had dropped precipitately and the patient experienced great subjective improvement. This was more striking in the younger than in the older patients.

Resolution of the pneumonic lesion as shown on roentgenograms was not noticeably different from that experienced with other forms of treatment used in recent years. The lungs of most patients cleared within about a week, though in some instances progress was slower. Representative films are presented in Figs. 1 and 2. Complications were conspicuously absent. Once treatment had been inaugurated meningitis, endocarditis, arthritis, or empyema did not



Fig 1—Bacteremic Type 3 pneumococcal pneumonia in a 3 year old woman treated on the 300 000 unit schedule  
Marked resolution of pulmonary lesion in six days



Fig 2—Nonbacteremic Type 7 pneumococcal pneumonia in a 29 year old man treated on the 300 000 unit schedule  
Complete resolution of pulmonary lesion in six days



ensue In two instances sterile pleural effusions appeared after recovery from the pneumonia, and in neither case did the effusion recur after its removal by thoracentesis

Three of the four deaths occurred in patients in the eighth decade The four fatal cases are summarized below

1 E G, CGH233031 This 79-year-old woman was admitted with the history of an acute febrile respiratory illness of a week's duration and chronic cough of several years' duration The temperature was 101.8° F, pulse 120, respirations 48, and blood pressure 180 systolic and 90 diastolic She was very ill, presenting signs of consolidation over the right upper lobe and evidence of patchy pneumonia in all other lobes In addition she suffered from decompensated arteriosclerotic heart disease

Sputum culture yielded pneumococcus Type 34, blood culture was negative Admission white blood count was 8,800

She was placed on the 300,000 unit penicillin schedule, given oxygen, and digitalized She responded with temporary improvement, but lost ground and died six days after admission Autopsy revealed evidence of chronic bronchiectasis and pulmonary fibrosis, bilateral lobular pneumonia, pulmonary edema, and hypertrophy of the right ventricle of the heart

2 T O'B, CGH235314 This 77-year-old white man was admitted to the hospital on Feb 20, 1948, in a critical state and died eight days later He had been ill for about three weeks, though no specific symptoms could date the onset of the pneumonia Temperature 100, pulse 90, respirations 22, blood pressure 130 systolic and 60 diastolic He was severely dehydrated and greatly undernourished Cyanosis was moderate There were signs of pneumonia in the right upper lobe, evidence of generalized arteriosclerosis and auricular fibrillation

Both sputum and blood cultures yielded Type 2 pneumococcus The white blood count was 6,000, the blood urea nitrogen 40 milligrams per cent

He was placed on the 300,000 unit schedule, and though blood and sputum cultures were negative on the third hospital day, he failed to improve clinically It seemed remarkable that he lived eight days

An autopsy revealed confluent lobular pneumonia involving the right upper, middle and lower lobes There was aspirated food in the left lower lobe with partial occlusion of the bronchus There was no meningitis or endocarditis

3 L V, CGH236791 This was a case of fulminating Type 2 pneumococcal pneumonia in a woman of 34 The patient was admitted to the hospital on April 1, 1948, with pneumonia of about three days' duration Temperature 102° F, pulse 120, respirations 28, blood pressure 106 systolic and 40 diastolic There were signs of consolidation in the right lower and middle lobes On her way to the ward, she went into shock She was placed on the 300,000 unit schedule and given 500 cc plasma and 2,000 cc 5 per cent glucose intravenously but died twelve hours later Permission for autopsy was denied

Cultures of sputum and blood yielded Type 2 pneumococcus The white cell count was 7,250 The blood urea nitrogen was 12 milligrams per cent

A. I. B., 66123755. This 74-year-old white man was admitted to the hospital on April 26, 1948, and died six hours later. The temperature was 101.4° F., pulse 120, respirations 28, and blood pressure 140 systolic and 90 diastolic. He presented the signs of decompensated atherosclerotic heart disease and pneumonia of the left lower lobe. He had been acutely ill for two weeks and received an unknown amount of sulfadiazine and penicillin before admission to the hospital.

Blood and sputum cultures yielded Type 15 pneumococcus. The white blood count was 32,350.

While on the ward he received one injection of 200,000 units of penicillin. Permission for autopsy was not obtained.

Only one death occurred among the fifteen cases of pneumococcal pneumonia treated with 25,000 units penicillin every three hours. This patient was a 94-year-old man suffering from Type 1 pneumococcus pneumonia who died on the eighth day of treatment. The only bacteremic patient in this series is covered. The mortality rate was 3.4 per cent.

There were no deaths among the fifty-four cases which were diagnosed as "pneumonia," but from which no pneumococci were recovered. Since several series of cases of "pneumonia" have been reported in the literature in which pneumococcus typing was not carried out, we have calculated the over-all mortality in our 118 cases of pneumococcal and nonpneumococcal pneumonia treated with 200,000 or 300,000 units a day for comparison with such series. The rate was 3.4 per cent.

#### DISCUSSION

The clinical results reported in this paper are of interest from the practical and theoretical points of view. In this time of shortage of nursing and other hospital personnel the advantages of administering penicillin only twice the first day and once a day thereafter are evident. There are also advantages in the use of crystalline penicillin free of procaine, beeswax, and oils of various types which though they have served us well do produce a certain number of undesirable side reactions.

The mortality rates—15 per cent in bacteremic, 2.3 per cent in nonbacteremic, and 6.3 per cent for bacteremic and nonbacteremic patients—are lower than have been encountered previously in the Cincinnati General Hospital for similar series of cases. Thus, in the years 1945-1946 and 1946-1947 the gross mortality in pneumococcal pneumonia was, respectively, 12.0 per cent and 14.6 per cent. The patients in these series were unselected. There was no exclusion because of age, state of sobriety, severity of illness, or duration of pneumonia.

It is not our intention in this place to discuss the interesting theoretical implications of this penicillin schedule, for they will be alluded to in a separate report dealing with penicillin blood levels in these patients<sup>11</sup>. Suffice it to say here that very high blood levels were found one hour after the initial dose of penicillin and measurable levels for at least six hours afterwards.

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Sputum culture yielded pneumococcus Type 34, blood culture was negative Admission white blood count was 8,800

She was placed on the 300,000 unit penicillin schedule, given oxygen, and digitalized She responded with temporary improvement, but lost ground and died six days after admission Autopsy revealed evidence of chronic bronchiectasis and pulmonary fibrosis, bilateral lobular pneumonia, pulmonary edema, and hypertrophy of the right ventricle of the heart

2 T O'B, CGH235314 This 77-year-old white man was admitted to the hospital on Feb 20, 1948, in a critical state and died eight days later He had been ill for about three weeks, though no specific symptoms could date the onset of the pneumonia Temperature 100, pulse 90, respirations 22, blood pressure 130 systolic and 60 diastolic He was severely dehydrated and greatly undernourished Cyanosis was moderate There were signs of pneumonia in the right upper lobe, evidence of generalized arteriosclerosis and auricular fibrillation

Both sputum and blood cultures yielded Type 2 pneumococcus The white blood count was 6,000, the blood urea nitrogen 40 milligrams per cent

He was placed on the 300,000 unit schedule, and though blood and sputum cultures were negative on the third hospital day, he failed to improve clinically It seemed remarkable that he lived eight days

An autopsy revealed confluent lobular pneumonia involving the right upper, middle, and lower lobes There was aspirated food in the left lower lobe with partial occlusion of the bronchus There was no meningitis or endocarditis

3 L V, CGH236791 This was a case of fulminating Type 2 pneumococcal pneumonia in a woman of 34 The patient was admitted to the hospital on April 1, 1948, with pneumonia of about three days' duration Temperature 102° F pulse 120, respirations 28, blood pressure 106 systolic and 40 diastolic There were signs of consolidation in the right lower and middle lobes On her way to the ward, she went into shock She was placed on the 300,000 unit schedule and given 500 cc plasma and 2,000 cc 5 per cent glucose intravenously, but died twelve hours later Permission for autopsy was denied

Cultures of sputum and blood yielded Type 2 pneumococcus The white cell count was 7,250 The blood urea nitrogen was 12 milligrams per cent

## NEW PENICILLIN PRODUCTS FOR SUSTAINED EFFECTS

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THESE studies were intended to devise penicillin preparations which would consistently yield prolonged and enhanced blood serum levels following a single subcutaneous or intramuscular injection. The ultimate objective was to develop a clinically effective, slowly and wholly absorbable, nontoxic product which could also be adapted for oral use.

Two major approaches were explored.

One approach was to encase crystalline salts of penicillin in protein, preferably of nonantigenic type such as blood serum of the same species or gelatin. The resulting penicillin-protein complex was then insolubilized with formaldehyde or with a heavy metal salt which formed insoluble derivatives with both penicillin and protein. The cations employed were those required by the body, namely ferric and cupric salts.

The other approach was to suspend these penicillin preparations or crystalline Na penicillin "G" in Tween 20 (polyoxyalkylene derivative of sorbitan monolaurate)\*. This was predicated on the hypothesis that Tween 20 retards penicillin excretion by attaching itself to lipids and lipoproteins at one end and to penicillin at the other end which results in formation of a larger molecule. The possibility of a Carouamide-like effect was also considered.

### METHODS

#### (A) Technique of Preparation of Formaldehyde Treated Penicillin Protein Mixtures—

1. 1 Gm. Na (or Cu) penicillin and 0.5 to 2.5 Gm. gelatin, in 15 ml.  $H_2O$ , are dehydrated while frozen.
2. The dried mixture is treated with 5 ml. of 40 per cent formaldehyde in 125 ml. acetone overnight, at 0 to 5°. Acetone is employed because in it penicillin is stable and both the penicillin and formaldehyde-treated gelatin are insoluble.
3. The penicillin-containing precipitate is filtered, dried in vacuo and finely ground.

#### (B) Preparation of $Cu^{++}$ or $Fe^{++}$ Penicillin Protein Mixtures—

1. To 0.6 Gm. penicillin dissolved in 15 ml. plasma 320 ml. of 2.5 per cent  $CuSO_4$ , 7  $H_2O$  or  $FeCl_3$  are added.
2. The penicillin-containing precipitate is filtered, washed with  $H_2O$ , dried in vacuo, and finely ground.

(C) For orientation purposes all products were first tested in animals. Experiments were carried out as a rule with single dosages equalling 300,000 O.U. penicillin. Potency controls assays were done by both biological and colorimetric methods. The insolubilized penicillin-protein complex first had to be trypsin digested in order to release the penicillin for assay.

(D) Blood samples were drawn periodically over a span of twenty-four to forty-eight hours for determination of penicillin levels. In evaluating some preparations the penicillin

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\*The Tween 20 was supplied by the Atlas Powder Company, Wilmington, Del.

content of the urine was also estimated in order to establish the extent and duration of penicillin elimination

It might be stated that our serum penicillin assay method employing *Streptococcus pyogenes* C203 as test organism yields generally and, at times, appreciably lower values than the Food and Drug Association assay method which uses the *Bacillus subtilis* as test organism.<sup>3,4</sup> Therefore, recorded data with the streptococcus C203 system, as a rule, are on the conservative side

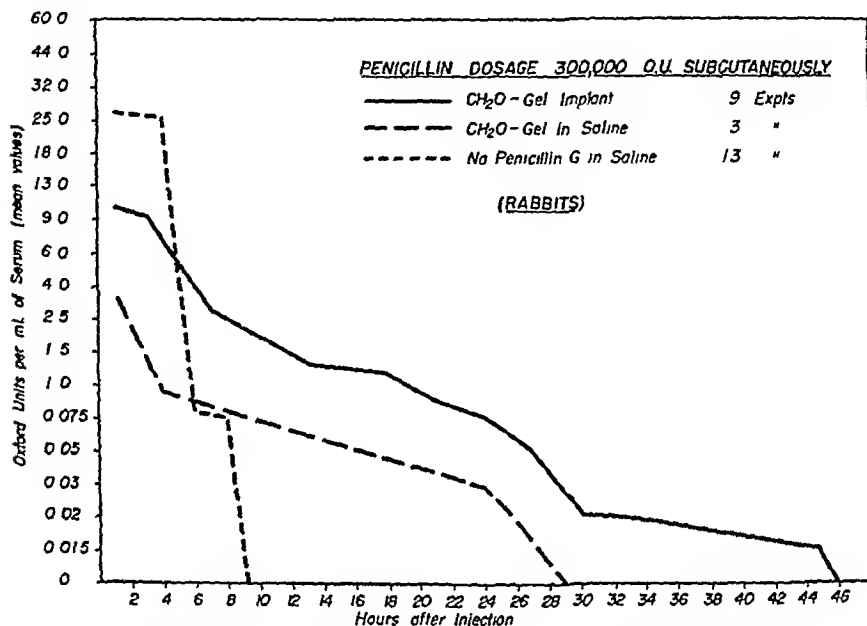


Fig. 1—Penicillin blood levels in rabbits following single subcutaneous dose of formaldehyde treated penicillin gels

#### EXPERIMENTAL OBSERVATIONS

At the outset the products were tested in rabbits by direct implantation under the skin in open gelatin capsules to obviate any influence of a vehicle. As may be seen in Fig. 1, the injection of crystalline Na penicillin "g" in saline fails to show measurable blood serum levels after nine hours. By contrast with these controls prolonged effects are noted up to forty-five hours with the penicillin-gel implants and up to twenty-four hours with the same products suspended in saline and injected subcutaneously.

The formaldehyde-treated penicillin gels\* were mechanically ground to particle sizes permitting injection through an 18 or 20 gauge needle. Because the resultant fine, insoluble, free-flowing powders tended to settle out in saline, they were suspended in ethylene-glycol and in Tween 20. While ethylene-glycol allowed ease of handling as compared with the gels suspended in saline, the measurable penicillin serum values were about the same, enduring for over twenty-four hours. However, the same gels suspended in Tween 20 reflected not only the stabilizing property but also the additive, prolonging, and enhancing effects of Tween 20, the composite result was marked prolongation of

\*Referred to in illustrations as CH O Gels

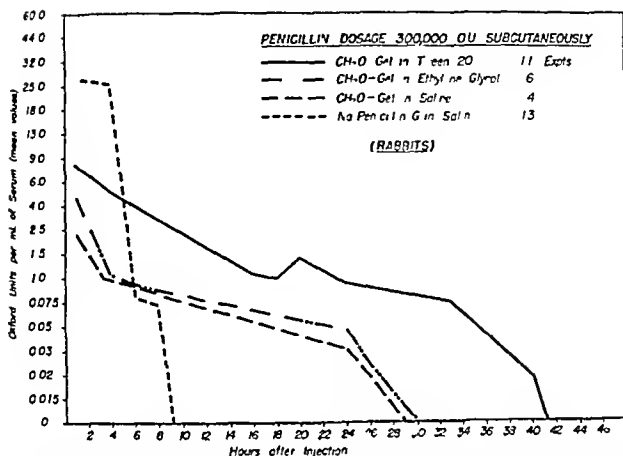


Fig. 2.—Comparative penicillin blood level in rabbits following single subcutaneous dose of formaldehyde treated penicillin G in various vehicles: ethylene glycol and Tween 20.

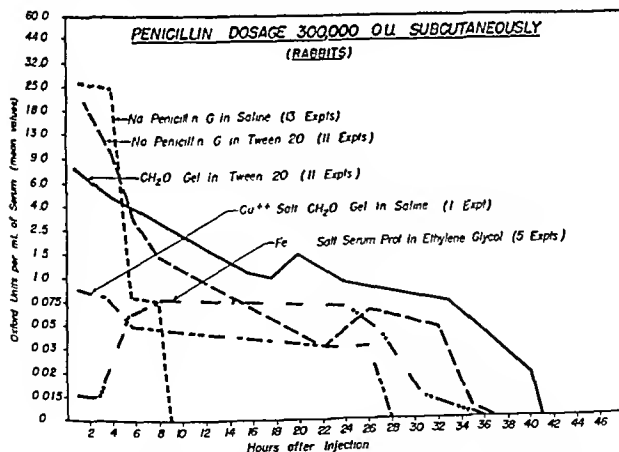


Fig. 3.—Penicillin blood levels in rabbits following single subcutaneous dose of metal penicillin protein mixtures.

detectable penicillin levels for a span of forty hours with relatively high values for thirty four hours (Fig. 2).

These singular effects were probably accomplished in a twofold manner, (1) the insolubilized penicillin protein complexes were slowly degraded in the

tissues by enzymatic action harmless to penicillin, and (2) the liberated penicillin combined with the Tween 20 which then exerted its augmenting action

Animal experiments were also conducted with copper- and iron-penicillin-protein mixtures (Fig 3) The penicillin values for these copper and iron complexes were compared with formaldehyde-treated gels suspended in Tween 20 The metal products yielded flatter curves suggesting that they dissociated less readily This is in keeping with published data<sup>5</sup> that metal penicillin products generally do not break down readily in vitro and dissociate but slowly in vivo Prolonging effects are nevertheless apparent, levels being present up to twenty-six hours with the copper and up to thirty hours with the iron products The formaldehyde-treated gels in Tween 20 still evidence the most conspicuous prolonging and enhancing effects

Comparison of graphs showing the average blood levels obtained after subcutaneous injections of 300,000 O U of penicillin in Tween 20 and in saline shows that Tween 20 per se has prolonging and enhancing properties, thirty-four hour detectable levels as against nine-hour control levels

In order to explore further this phenomenon and elucidate its mechanism, animal experiments were done as recorded in Table I Each experiment was carried out with a single dose of 500,000 O U crystalline Na penicillin "G"

TABLE I PENICILLIN SUSTAINING AND/OR ENHANCING EFFECTS OF TWEEN 20 IN RABBITS

EXPERIMENT	BLOOD LEVELS OXFORD UNITS PER ML OF SERUM							
	16 HR	20 HR	24 HR	28 HR	32 HR	40 HR	48 HR	67 HR
Control—5 ml Tween 20 sc	0	0	0					
Repeat in 3 hours sc								
Control—Na Penicillin G 500,000 O U in saline sc	0	0	0					
Na Penicillin G 500,000 O U in 5 ml Tween 20 sc	15		05	05	075		015	
5 ml Tween 20 sc r flank, 3 hours later Na Penicillin G 500,000 O U sc l flank	15	15	0375			0		
5 ml Tween 20 sc, repeat in 3 hours sc, followed in 3 hours by Na Penicillin G 500,000 O U in saline sc	375		37	0375		075	05	015

It was evident from these studies in rabbits that

- 1 Tween 20 subcutaneously had of itself no antibiotic effects
- 2 Penicillin in saline subcutaneously gave no detectable levels at and after sixteen hours
- 3 Injection of the penicillin mixed in Tween 20 yielded appreciable levels for forty-eight hours

4 The prolonging property of Tween 20 was observed even though it was injected separately three hours before the penicillin and at a different site. Substantial penicillin levels were obtained for twenty four hours.

5 The combination of Tween 20 and penicillin necessary for prolonging and enhancing effects may occur in the blood as shown in the last of this series of tabulated animal experiments. Here two separate subcutaneous injections of Tween 20 were given three hours apart, six hours after the first and three hours after the second Tween 20 injection, penicillin in saline was given intravenously. Prolongation was stilling and persisted for sixty seven hours.

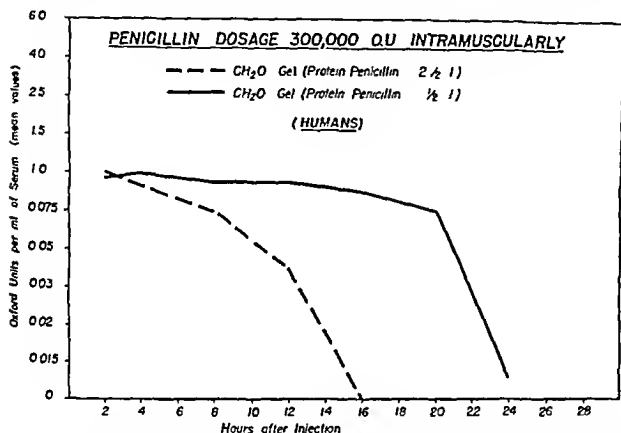


Fig 4—Influence of protein content of formaldehyde treated penicillin gels on sustaining blood levels following single intramuscular dose in human beings

The principles embodied in these various preparations have been applied in human subjects. While our studies in this direction are by no means complete, similar prolonged effects were noted following a single dose injection of insolubilized penicillin gel suspended in saline or Tween 20 and a similar dose injection of Na penicillin "G" in Tween 20.

In the case of the formaldehyde treated penicillin gel it was not possible to translate the results of animal experimentation into clinical use without revision of the preparation. For instance, in the matter of the protein content it was found that when the proportion of protein to penicillin was maintained at 2 1/2 to 1, penicillin could be detected in body fluids, because of retarded degradation of the protein penicillin complex for as long as ninety six hours after the injection which was as far as the tests were pursued. Under these circumstances detectable blood levels were maintained for only twelve hours. However, when the proportion of protein to penicillin was reduced to 1/2 to 1, detectable blood levels of a relatively high order were maintained for twenty four hours (Fig 4). Apparently the reduction in protein content resulted in release of the penicillin at a more rapid and even tempo.



The penicillin sustaining and/or enhancing properties of Tween 20 were also manifested in human subjects during the following series of experiments (Table II)

1 Crystalline Na penicillin "g," 500,000 O U in saline injected intramuscularly gave no detectable blood levels at eight hours or thereafter

2 Crystalline Na penicillin "g," 500,000 O U dispersed in 5 ml of Tween 20 and injected intramuscularly yielded sustained and enhanced effects for twenty hours

3 In this experiment the patient was given 5 000 000 O U of Na penicillin daily by continuous venoclisis. Control blood assays on this dosage schedule were 5 O U per milliliter of serum. Tween 20 was given orally, 10

TABLE II PENICILLIN SUSTAINING AND/OR ENHANCING EFFECTS OF TWEEN 20 IN HUMAN BEINGS

EXPERIMENT	BLOOD LEVELS OXFORD UNITS PER ML OF SERUM							
	2 HR	4 HR	6 HR	8 HR	12 HR	16 HR	20 HR	26 HR
Control—Na Penicillin G 500,000 O U in saline i m		10		0.012	0	0		
Na Penicillin G 500,000 O U in 5 ml Tween 20 i m		15		0.75	0.36	0.08	0.03	0
Na Penicillin G 5,000,000 O U in saline 24 hr continu- ous i v, 50 ml Tween 20 orally in divided doses	50	75		150	150	150	350	50
CH <sub>2</sub> O Gel 500 000 O U orally, 50 ml Tween 20 orally in divided doses	0.75	0.15	0.08	0.05	0.04	0.04		

ml every four hours for five doses. An enhancing effect was observed beginning four hours after and persisting for twenty hours after the last dose of Tween 20 when it reached a peak level of 35 O U per milliliter of serum which was seven times the control value.

4 In the final experiment of this series, 500 000 O U of a protein penicillin complex were given orally in the morning on a fasting stomach. Ten milliliters of Tween 20 were given orally every two hours for the duration of the experiment. Therapeutic penicillin blood values were obtained throughout the entire sixteen hours of this test.

Further studies were carried out with oral administration of formaldehyde treated penicillin products. Data thus far indicate that detectable levels up to twenty-four hours have been obtained following oral administration of 300,000 O U of the protein-penicillin complex with and without the conjoint use of Tween 20. As indicated by *in vitro* tests, the insolubilized protein-penicillin complex is gradually digested by trypsin which has no destructive influence on the slowly liberated penicillin\*. These investigations are still being pursued in

\*The oral use of Tween yielded additional prolongation effects. This was probably achieved by retarding the excretion of penicillin in the manner previously suggested.

an effort to develop a consistently effective predictable long acting medium for the oral administration of penicillin

The factors responsible for this prolonging and enhancing action of Tween 20 in both rabbits and human subjects are still being investigated as are other facets of this problem. It is of interest to note that comparable studies with heparin show similar results these will be presented later in detail. Studies thus far indicate that the Tween effect is not due to impairment of renal function in the usual sense. Tween 20 and a limited compound (polyoxalkylene derivative of mannitan monolaurate) were found to be innocuous in long term feeding experiments in animals and no toxic manifestations were reported following its parenteral and oral use in human beings. No side effects were observed in human subjects after ingestion of capsules containing the protein penicillin products or following Tween taken orally in divided doses totaling 100 milliliters.

#### SUMMARY

Prolongation of serum penicillin levels were observed in rabbits and human beings following single subcutaneous or intramuscular injections of formaldehyde treated protein penicillin preparations. Significant sustaining and enhancing effects were noted following the administration of the insolubilized protein penicillin complexes or crystalline Na penicillin G suspended in Tween 20. The mechanism of this property of Tween 20 was investigated. Measurable penicillin levels were obtained in human subjects up to twenty four hours following ingestion of a single dose of penicillin G, alone or in combination with Tween. Further studies are in progress with the ultimate objective of developing practical penicillin sustaining and enhancing products for subcutaneous intramuscular and oral use in human beings.

The authors are indebted to Mr. Sidney Noble for technical assistance.

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## THE TURBIDIMETRIC ASSAY OF HYALURONIDASE

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THE various methods suggested for the quantitative determination *in vitro* of the enzyme hyaluronidase are dependent on its property of hydrolyzing hyaluronic acid. During hydrolysis, the reducing properties of a solution of hyaluronic acid are increased, the viscosity is decreased, and as observed by Meyer and Palmer,<sup>1</sup> the ability of hyaluronic acid to precipitate with acidified protein is decreased. These observations served as the basis of the viscosity test, the mucin clot test, and the turbidimetric assay method for hyaluronidase activity. A comprehensive review of the entire problem of hyaluronidase and hyaluronic acid, including a discussion of the assay techniques, has been prepared recently by Meyer.<sup>2</sup>

The present investigation deals with the turbidimetric method of assay which has been developed by Seastone,<sup>3</sup> Kass and Seastone,<sup>4</sup> Leonard, Perlman, and Kurzrok,<sup>5</sup> and Dorfman and Ott.<sup>6</sup> We have studied various factors which might influence the sensitivity and accuracy of the assay in order to obtain optimal conditions for hydrolysis and development of turbidity. On the basis of these studies it has been possible to devise a more satisfactory technique for the turbidimetric assay of hyaluronidase and to define a unit for the enzyme.

### A. METHOD AND MATERIALS

The principles of the assay technique used in the first part of this study were essentially those described by Meyer.<sup>2</sup> Enzyme and substrate were incubated for thirty minutes at 37.5° C in an acetate sodium chloride buffer at pH 6. Turbidities were then developed by diluting the system with pH 4.2 acetate buffer and adding acidified protein solution. The turbidities were read in a Summerson Klett photoelectric colorimeter which had been standardized in terms of light transmission against a Beckman spectrophotometer. The Klett readings, obtained with a blue filter, can be expressed as percentage transmission for wave length 680 mμ by using the following formula: % transmission =  $94.53 - (0.197 \times \text{Klett reading})$ . The results of the assays were expressed in Turbidity Reducing Units (TRU) tentatively defined as the amount of enzyme which will reduce the turbidity produced by 0.2 mg of hyaluronic acid to that produced by 0.1 mg of hyaluronic acid in accordance with previous authors.<sup>2, 4</sup> This definition was used during the experimental part of this paper which led to the establishment of a new unit.

Hyaluronic acid was prepared from human umbilical cords by a modification of the method of Rogers.<sup>7</sup> Both sodium and potassium salts were used in these studies. Hyaluronidase was prepared from bovine testes by ammonium sulfate fractionation. Acidified proteins were prepared from various sources. Serum or plasma was diluted with 9 parts of 0.5M acetate buffer at pH 4.2 and adjusted to pH 3.1 with 4N HCl.

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## B ENZYME SUBSTRATE REACTION

*1 Influence of Sodium Chloride*—It has been noted that the apparent enzymatic activity of testicular hyaluronidase depends greatly on the nature and concentration of the electrolytes present. In a given buffer system sodium chloride of certain concentrations seems to have a specific accelerating influence on the velocity of this enzymatic reaction.<sup>8,9,10</sup> We therefore studied the influence of sodium chloride on the activity of hyaluronidase. A preparation of enzyme was used which gives an assay of 27 TRU per milligram when incubated with substrate in 0.1M acetate buffer pH 6 containing physiologic saline (0.15M). Using pH 6 acetate buffer with varying amounts of sodium chloride, we found that the apparent units per milligram of hyaluronidase ranged from 5 in the absence of sodium chloride to 13 in the presence of 0.07M sodium chloride. At higher concentrations of sodium chloride the apparent enzymatic activity decreased. It is desirable that the assay values of the enzyme should reflect the activity of the enzyme under physiologic conditions. Since physiologic salt solution (0.15M) gives very satisfactory assay values this concentration of sodium chloride has been employed.

*2 Heat Inactivation*—In order to avoid further hydrolysis of hyaluronic acid after the thirty minute period of incubation with hyaluronidase the reaction was stopped by immersing the tubes in a 60° C. water bath for ten minutes. It was found that this temperature was sufficient to inactivate hyaluronidase solutions.

## C DEVELOPMENT OF TURBIDITY

*1 Stabilization of Proteins by Aging*—The assay method as originally published called for the use of horse serum as the protein indicator to measure the quantity of hyaluronic acid remaining after enzymatic hydrolysis. Our observations indicated that the age of the protein solution was much more important than had been recognized by earlier workers.<sup>2</sup> We found that immediately after diluting fresh serum there was no consequential difference between the turbidity caused by a given amount of this serum with 0.1 mg. and 0.2 mg. of hyaluronic acid. Therefore assays performed with freshly diluted serum will not have the desired degree of accuracy. It was noted however, that after standing in the cold for some time, the same acidified serum would give greater turbidities with hyaluronic acid, and that the spread between the blanks also increased with time. The effect of aging over a period of three weeks was therefore investigated.

In this study we used, as the source of protein, freshly collected as well as commercially available horse serum, human serum obtained from healthy volunteers and human plasma purchased from Cutter Laboratories. In a search for a more stable protein, we included in this investigation such purified protein fractions as crystalline horse serum albumin prepared by the method of Svedberg and Sjogren,<sup>11</sup> crystalline egg albumin prepared by the methods of Cole<sup>1</sup> and Longworth and co-workers<sup>12</sup> and Fraction V of bovine plasma albumin, prepared by Armour Laboratories according to the method of Cohn.<sup>14</sup> The

TABLE I INFLUENCE OF AGING AND HEATING OF DILUTE PROTEIN SOLUTIONS (1:10) ON TURBIDITY READINGS AND APPARENT ACTIVITY OF HYALURONIDASE FRACTION HD38 99 VIII

SAMPLE	PROTEIN SOL'N AGED IN 11F °C/D										PROTEIN SOL'N AGED BY HEATING TO 100 °C										WJRAU TRU/ MG	
	AGE	BLANKS					TRU/MG	AGE	BLANKS					TRU/MG	Δ	BLANKS						TRU/MG
		0.2 MG		0.1 MG		Δ			0.2 MG		0.1 MG		Δ			0.2 MG		0.1 MG		Δ		
<i>A Horse Serum</i>																						
Local	1 hr *	114	92	92	22	100	100	2 days	228	133	95	46	46	46	46	228	133	95	46	46	46	
Local	2 days*	118	94	24	24	118	118	Fresh	298	157	98	45	45	45	45	298	157	98	45	45	45	
Lederle	Fresh	101	95	16	16	135	135	1 day	245	160	145	35	35	35	35	245	160	145	35	35	35	
Lederle	1 day	100	85	15	15	154	154	20 days	215	142	104	44	44	44	44	215	142	104	44	44	44	
Lederle	20 days	189	123	66	66	46	46	Fresh	216	142	104	44	44	44	44	216	142	104	44	44	44	
Parke, Davis I	Fresh	181	127	54	54	95	95	20 days	234	142	104	44	44	44	44	234	142	104	44	44	44	
Puke, Davis I	20 days	177	120	57	57	59	72	Fresh	250	138	112	41	41	41	41	250	138	112	41	41	41	
Parke, Davis II	Fresh	190	131	59	59	72	72	7 days	234	138	106	44	44	44	44	234	138	106	44	44	44	
Parke, Davis II	7 days	192	122	70	70	61	61	21 days	235	139	99	44	44	44	44	235	139	99	44	44	44	
Parke, Davis II	21 days	203	128	75	75	61	61	21 days	243	142	101	48	48	48	48	243	142	101	48	48	48	
<i>B Human Serum</i>																						
Serum I	1 day	161	103	58	58	83	83	1 day	240	148	92	35	35	35	35	240	148	92	35	35	35	
Serum VI	Fresh	102	83	19	19	100	100	Fresh	297	152	78	36	36	36	36	297	152	78	36	36	36	
Serum VI	7 days	113	92	31	31	5	5	7 days	230	152	78	36	36	36	36	230	152	78	36	36	36	
Serum VI	21 days	-	100	-	-	36	36	21 days	299	148	80	32	32	32	32	299	148	80	32	32	32	
Serum VII	Fresh	154	106	48	48	72	72	21 days	236	135	102	29	29	29	29	236	135	102	29	29	29	
Serum VII	7 days	173	104	69	69	51	51	7 days	254	157	97	34	34	34	34	254	157	97	34	34	34	
Serum VII	21 days	181	104	77	77	31	31	21 days	218	142	95	22	22	22	22	218	142	95	22	22	22	
Serum VIII	Fresh	155	108	47	47	67	67	Fresh	242	149	108	18	18	18	18	242	149	108	18	18	18	
Serum VIII	7 days	154	102	52	52	51	51	7 days	249	161	85	31	31	31	31	249	161	85	31	31	31	
Serum VIII	21 days	185	112	71	71	49	49	21 days	252	162	90	29	29	29	29	252	162	90	29	29	29	
Serum IX	Fresh	120	90	30	30	80	80	7 days	250	151	99	28	28	28	28	250	151	99	28	28	28	
Serum IX	7 days	127	90	37	37	51	51	21 days	244	142	102	26	26	26	26	244	142	102	26	26	26	
Serum IX	21 days	140	98	42	42	75	75	21 days	239	149	90	31	31	31	31	239	149	90	31	31	31	
									220	140	80	40	40	40	40	220	140	80	40	40	40	
									229	149	80	42	42	42	42	229	149	80	42	42	42	
									226	128	98	32	32	32	32	226	128	98	32	32	32	

<i>C Human Plasma</i>									
Pla m <sub>1</sub> I	2 days	162	112	40	40	7 days	22	15	72
Pla m <sub>1</sub> I	Free h	136	111	43	42	Free h	41	15	71
Pla m <sub>1</sub> II	-1 days	134	119	66	51	21 days	28	11	95
Pla m <sub>1</sub> III	Free h	131	108	43	77	Free h	-1	15	1
Pla m <sub>1</sub> III	7 days	164	105	56	42	7 days	-1	140	5
Pla m <sub>1</sub> III	21 days	176	117	61	49	-1 days	-36	14	8
<i>D Protein Fractions</i>									
HS <sub>1</sub> †	2 days	221	166	32	31	2 days	-	14	33
HS <sub>1</sub> †	Free h	228	190	65	48	Free h	-33	17	37
HS <sub>1</sub> †	2 days	-	187	-	41	2 days	-	176	44
HS <sub>1</sub> †	7 days	204	184	80	40	7 days	-16	166	80
HS <sub>1</sub> †	21 days	231	174	37	38	-1 days	-16	160	7
BPV§	Free h	42	27	5	-	Free h	-5	140	17
BPV§	7 days	125	108	17	36	7 days	-23	137	19
BPV	-1 days	215	149	60	32	-1 days†	-80	147	19
							-91	155	20

After bleeding

† Age since bled

‡ Crystalline horse serum albumin

§ Bovine plasma albumin fraction V

sera and plasma were diluted and acidified to pH 3.1 as mentioned. Crystalline horse serum albumin and egg albumin were dissolved in buffer containing 0.5M acetate, pH 4.2 and 0.1M mandelic acid as stabilizer<sup>15</sup> at a concentration of 10 mg per milliliter and then adjusted to pH 3.1. With Fraction V of bovine plasma albumin, it was necessary to use a concentration of 20 mg per milliliter in order to get satisfactory turbidities. The solutions were kept at 5° C during the period of study.

Numerous assays of hyaluronidase were performed, using as indicators the acidified protein solutions (a) made up freshly, (b) after standing in the cold for one week, (c) after standing in the cold for three weeks. These experiments are recorded in the left columns of Table I which includes the turbidities obtained with hyaluronic acid (0.2 mg and 0.1 mg blanks) incubated in the absence of enzyme, the difference  $\Delta$  between the blanks, and the assay results in TRU per milligram. With locally collected horse serum, human serum, and Lederle horse serum, none of which contained a preservative, the spread between the turbidities of the blanks increased progressively with age. At the same time, the apparent units per milligram decreased. Similar changes were observed to a lesser degree with Parke, Davis horse serum, pooled human plasma and crystalline horse serum albumin. It therefore appeared that these proteins were already partially stabilized\*. On the other hand, the changes in the turbidities of the blanks obtained with bovine plasma solution aged in the cold for seven and twenty-one days were most striking. Aging by this procedure failed to give sufficient stabilization of the protein reagent to assure uniform assay results.

Crystalline egg albumin proved to be so unstable under the conditions of our experiments that its use had to be discontinued.

*2 Stabilization of Proteins by Heating*—Since aging in the cold did not fully stabilize the acidified proteins within a period of three weeks, an effort was made to accelerate the aging process by heating. The results are included in the right columns of Table I. Aliquots of the solutions used in the study on aging were heated to 100° C on the days indicated in the table and used for assays. In several instances, the heated solutions were kept at 5° C for seven and twenty-one days respectively and then used for assays.

The results indicate that the heated proteins gave considerably higher turbidity readings with hyaluronic acid (0.2 mg and 0.1 mg blanks) than observed with the unheated proteins. The differences between the turbidities of the blanks were much greater and showed less variation. The units per milligram calculated from these blanks were lower but much more constant (see Table I). Protein solutions, once heated, gave the same turbidities and assay results with hyaluronic acid after standing in the cold for seven and twenty-one days respectively. It should be mentioned that the bovine plasma albumin solutions had been stabilized by the improved heating technique described below which results in a lowering of the assay values.

\*Parke Davis horse serum contained 0.4 per cent purified cresol and the human plasma contained 5 per cent dextrose and 1:15,000 phenylmercuric benzoate.

TABLE II HEAT STABILIZATION OF ACIDIFIED HUMAN PLASMA

NO	UNHEATED PLASMA			HEATED TO 80° C			HEATED TO 90° C			HEATED TO 99° C			HEATED 10 MIN. AT 99° C			HEATED 10 MIN. AT 99° C					
	BLANKS	TRU/ MG		TIME	BLANKS	TRU/ MG	TIME	BLANKS	TRU/ MG	TIME	BLANKS	TRU/ MG	TIME	BLANKS	TRU/ MG	TIME	BLANKS	TRU/ MG			
1	104	102	50	150	240, 148	35	3 10	-44	136	12	10 20	234	160	39	-11	130	33	-30	151	31	
2	106	115	67	25	234	151	44	4 20	242	157	41	-1 10	233	155	35	240	156	34	-38	154	31
3	166	114	57	215	240, 155	59	3 10	231	162	30	23 30	-17	158	30	244	154	26	218	150	20	
4	118, 89	80	25	"	212, 150	56	3 10	215	151	31	9 20	227	155	44	226, 152	34	230	145	29	230	145
5	120, 98	118	145	"	218, 144	44	4 15	222	151	41	9 10	273	166	40	212	160	34	210	160	30	
6	147, 112	80	145	"	240, 160	46	3 20	236	170	41	9 10	-44	161	33	252	158	28	248	164	26	
7	138, 105	100	25	"	232, 152	39	3 20	238	162	38	12 20	239	152	31	278	158	29	242	158	26	
8	130	109	91	20	232	161	44	3 20	240	164	36	10 20	243	172	36	218	176	33	234	172	28
Averages																					
	143	106	80	9 10	232	153	43	3 41	236	159	40	13 20	239	160	36	242	158	31	244	157	29

Experiments 1 to 7 carried out with human plasma #1

Experiment 8 carried out with human plasma #3

Experiments 1 4 and 5 heated on hot plate #1

Experiments 2 and 3 heated on hot plate #2

Experiments 6 to 8 heated on steam bath



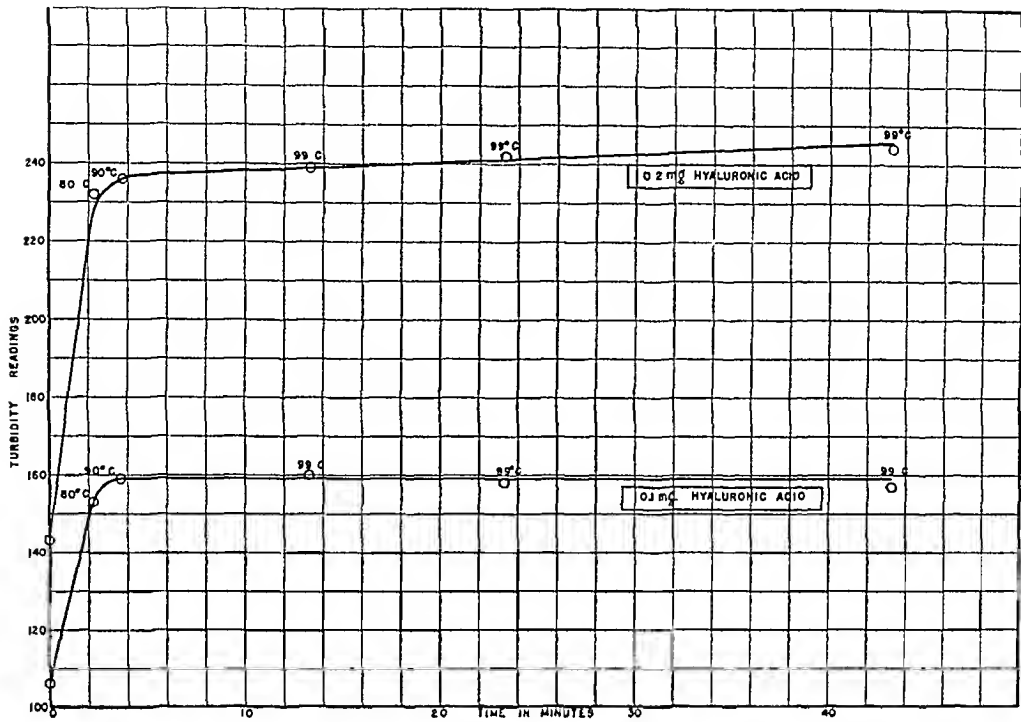


Fig 1—Influence of heat-stabilization of acidified plasma on turbidities obtained with hyaluronic acid.

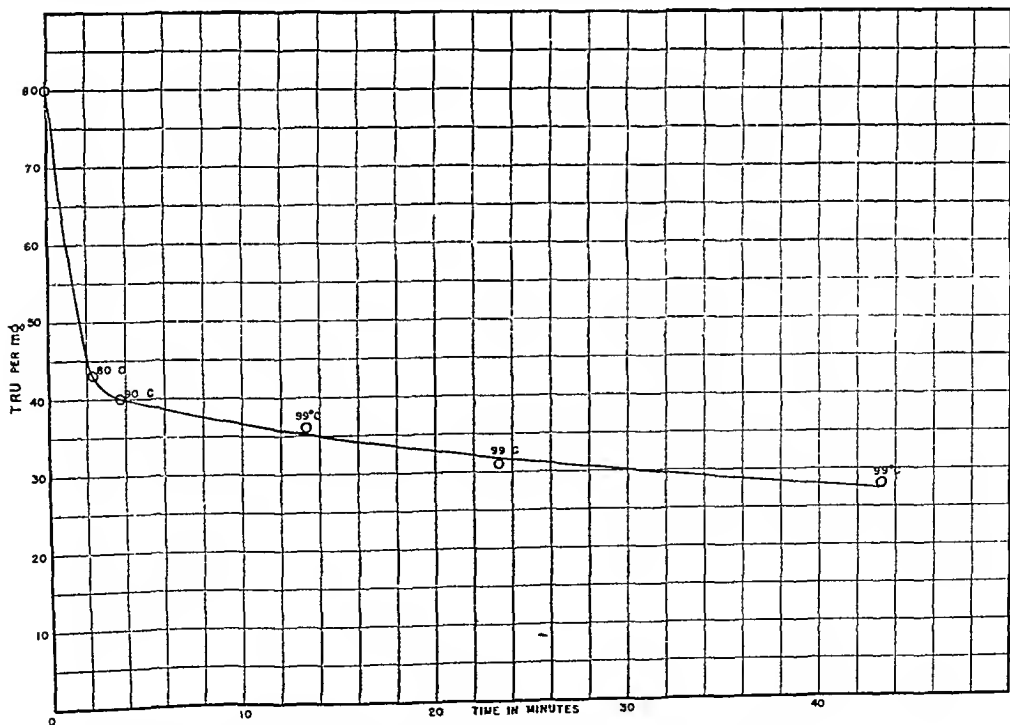


Fig 2—Influence of heat-stabilization of acidified plasma on apparent enzymatic activity of hyaluronidase

Because it is readily available to most investigators, all subsequent experiments have been carried out with human plasma

**3 Time Factor in Heat Stabilization**—From the results obtained it appeared that heated proteins were quite suitable for developing turbidities with hyaluronic acid. In order to further reduce the variations in the results the heat stabilization of protein solutions was studied in greater detail. Human plasma diluted and acidified to pH 3.1 as described was chosen for this investigation. In the previous experiments the plasma had been brought to a boil directly on an electric hot plate. Since this technique may not be entirely reproducible samples of plasma were heated under various controlled conditions and for different lengths of time. In all experiments 50 ml of diluted acidified plasma were placed in each of six test tubes measuring 25 by 200 mm. In each instance one sample of plasma was used without heating and the other samples were heated for various lengths of time. In the first five tests (Table II) the tubes with the plasma were heated in a 2 liter beaker containing 1 liter of boiling water. The other tests were performed by immersing the tubes containing plasma in a large steam heated water bath. The second procedure appeared in general to cause more rapid heating. At the completion of various periods of heating the tubes were removed from the bath and chilled. The protein solutions were then used as indicators in the determination of hyaluronic acid. The data obtained from eight heating experiments are summarized in Table II and the averaged results are illustrated in Figs. 1 and 2.

It can be seen from Fig. 1 that the unheated plasma proteins gave very low turbidities with hyaluronic acid. After heating however the proteins precipitated more readily with the substrate and the difference in the turbidities of the two blanks increased with the length of time of heating. Fig. 2 represents graphically the progressive decrease in the units per milligram under these conditions. The apparent enzymatic activity dropped sharply when plasma heated to 80 and 90° C was used as a protein indicator. With prolonged heating however, the curve leveled off. It may be concluded that plasma heated at 99° C for thirty minutes gives the most desirable results both in regard to the spread of the turbidities of the blanks and the accuracy of the assays.

**4 Influence of Hydrogen Ion Concentration on Heat Stabilization of Acidified Proteins**—Human plasma was diluted 1:10 with pH 4.2 acetate buffer, adjusted to hydrogen ion concentrations varying from 2.5 to 4.2 and heated as described. Assays were performed using these protein solutions as indicators. The final pH of the solutions in which turbidities were developed was found to vary only from 4.12 to 4.18.

Human plasma adjusted to pH 4.2 proved completely unsatisfactory in our hands because it coagulated upon heating. In this connection it is interesting to note that diluted horse serum could be heated at pH 4.2 without coagulating. However the blanks obtained with horse serum adjusted to pH 4.2 were too low and too close together to give the greatest possible accuracy.

The experiments carried out with human plasma at pH 2.5, 3.1, and 3.7 are shown in Fig. 3. Using plasma heated at these hydrogen ion concentrations

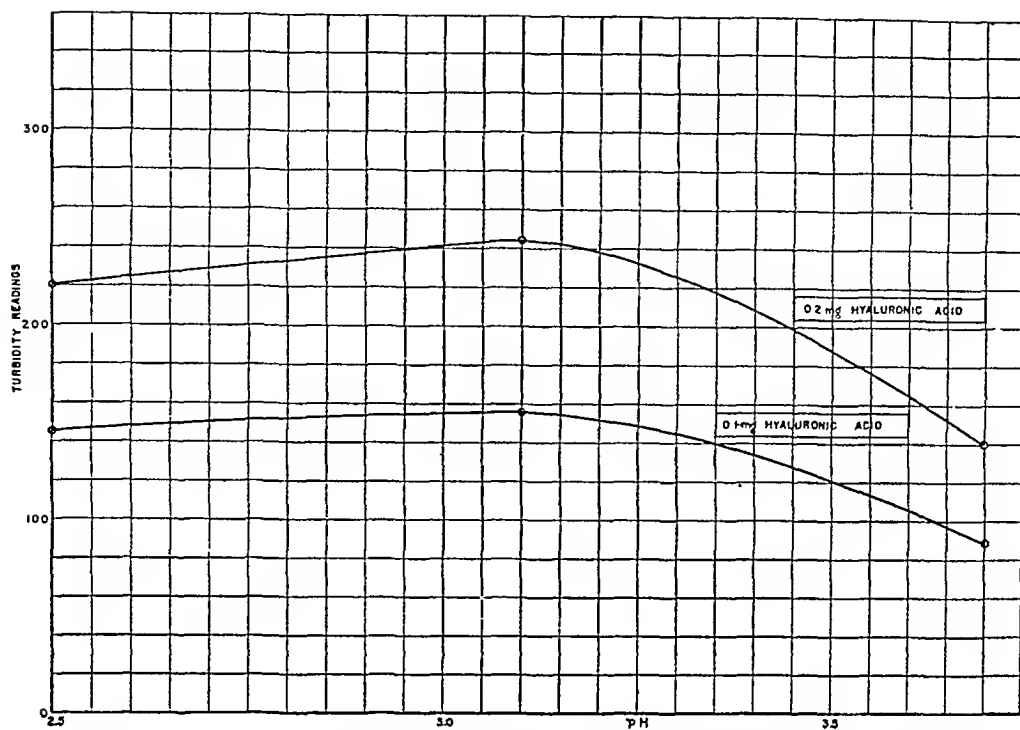


Fig 3.—Turbidities obtained with hyaluronic acid and human plasma heat-stabilized at different hydrogen-ion concentrations

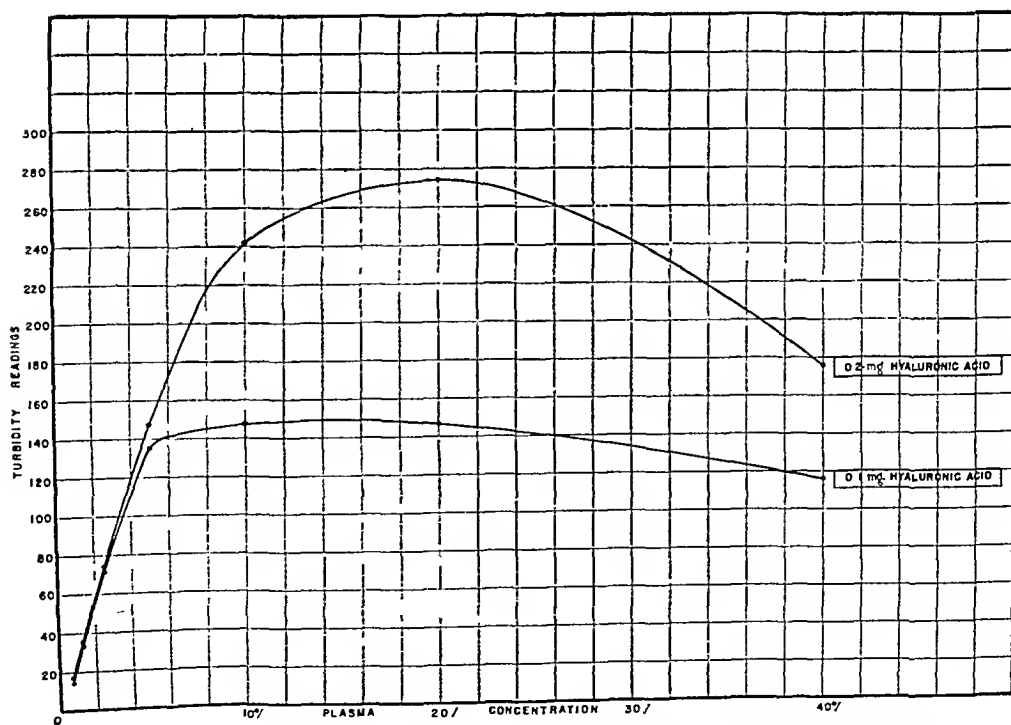


Fig 4.—Influence of plasma concentration on turbidities obtained with hyaluronic acid

the blank values for both concentrations of hyaluronic acid showed a maximum turbidity with plasma heated at pH 3.1. Also, the spread between the blanks was at a maximum at this pH. Thus hydrogen ion concentration may, therefore be considered most suitable for stabilizing plasma proteins. The assay results for the hyaluronidase fraction used gave 33, 34, and 20 TRU per milligram with plasma heated at pH 2.5, 3.1, and 3.7 respectively.

*5 The Influence of Protein Concentration on the Turbidity produced by Hyaluronic Acid*—The turbidity produced in the assay procedure depends not only on the amount of hyaluronic acid but also on the amount of protein in the system. With this in view, we have added various concentrations of heat stabilized plasma to hyaluronate in a buffer system identical to that used in the assay. The results are presented graphically in Fig. 4. Plasma was used at dilutions varying from 1:25 to 1:160 or 40 per cent to 0.625 per cent concentrations. Curves are plotted of the turbidities obtained with these protein solutions and 0.1 mg and 0.2 mg of hyaluronic acid. In both instances, it was found that the turbidity reached a maximum and then decreased upon the addition of more plasma. Whether the decrease in turbidity in the presence of excess plasma is the result of alteration in particle dispersion or increased solubility of the precipitate is not known. The maximum turbidity using 0.2 mg of hyaluronic acid is produced with plasma diluted 1:5, the maximum turbidity using 0.1 mg of hyaluronic acid is produced with plasma diluted 1:10.

#### D. SUBSTRATE

*1 Preparation of Hyaluronate From Umbilical Cords*—Human cords were washed free of blood and collected under 2 per cent aqueous phenol. They were removed from the phenol solution, passed once through a meat grinder and twice through an Eppenbach colloid mill and weighed. Two and one half kilograms of ground material were extracted three times for twenty-four hours with 9, 6, and 6 liters of 1 per cent aqueous phenol respectively. The crude hyaluronate was precipitated from the extracts in the form of a mucin clot by adding acetic acid to a concentration of 0.2 per cent. The precipitate was dried with alcohol and acetone and extracted with alkaline water at pH 8 (KOH or NaOH) at a concentration of 0.5 per cent. The extract was precipitated with  $1\frac{1}{2}$  volumes of denatured alcohol saturated with acetate, and dried. The alkaline extraction and alcohol precipitation were repeated once more.

In order to remove the proteins still present, the second alcohol precipitate was dissolved in water at pH 8 to a concentration of 0.5 per cent and shaken with 5 per cent by volume of a 1:1 mixture of chloroform butanol and centrifuged. This procedure was repeated with fresh chloroform butanol until no more protein was precipitated upon centrifugation. For further clarification, the solution was shaken with bentonite, centrifuged and precipitated with 2 volumes of denatured alcohol to which sodium or potassium acetate was added in amounts sufficient to induce flocculation. The yield was approximately one half gram per kilogram of ground cords.

TABLE III ANALYTIC COMPARISON OF SUBSTRATES PREPARED FROM UMBILICAL CORDS

FRACTION	ANALYTIC DATA					REL VISCOSITY*
	N (%)	K (%)	NA (%)	S (%)	MOISTURE (%)	
VIT 129 IV	3.52	10.6	—	Neg	9.22	10.98
VIII 3 IV	3.12	8.8	—	Neg	9.80	9.85
STI 35 IV	3.44	11.1	—	Neg	10.40	5.44
STI 50 IV	2.45	—	16.25	Trace	14.70	9.28

\*0.1 per cent solution in H<sub>2</sub>O relative viscosity of water = 1.00

2 *Properties of the Substrate*—Analytic data for various substrate batches prepared from umbilical cords are given in Table III. The preparations were chemically similar, the average nitrogen was 3.13 per cent, the average potassium calculated as potassium sulfate was 10.1 per cent. The viscosities varied from 5.4 to 11. The turbidities produced with stabilized plasma were all of the same

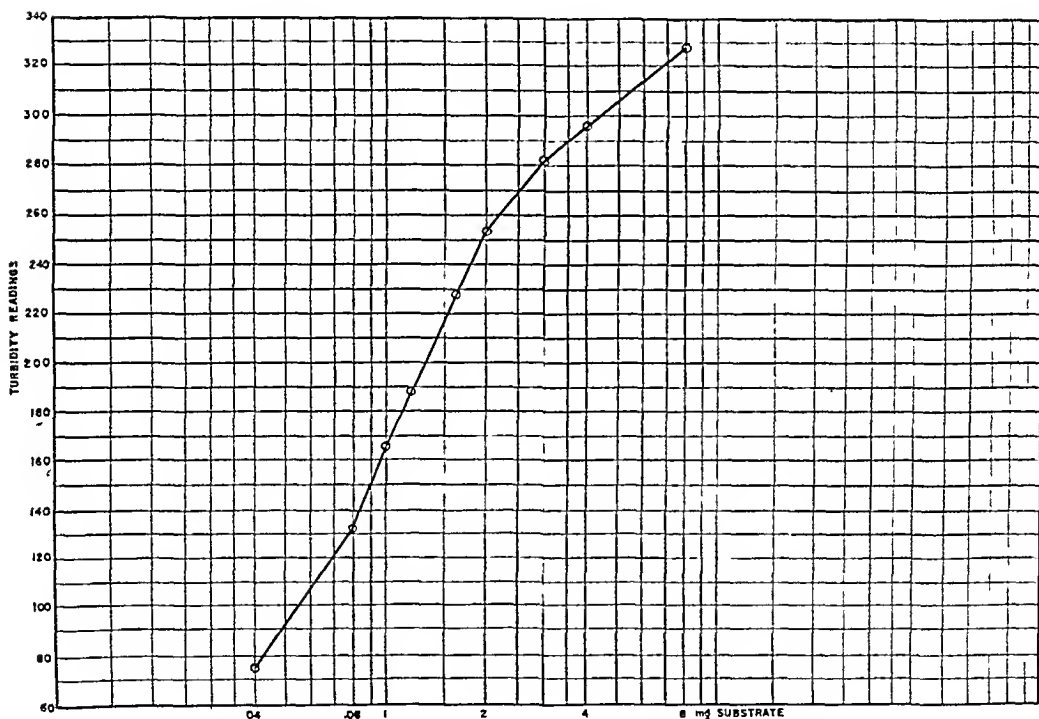


Fig. 5—Standard curve of four substrate preparations

magnitude but it has been our experience that the variations from batch to batch of hyaluronate are such that standard curves must be prepared for each batch. A standard curve obtained with 0.04 to 0.8 mg of hyaluronate and a constant amount of human plasma stabilized at 99° C for thirty minutes is demonstrated in Fig. 5. When plotted on semilogarithmic paper, the standard curve is S shaped as observed before by Meyer.<sup>2</sup> It gave a straight line for the turbidity readings between 250 and 125 which, in terms of light transmission, corresponds to a range of 45 to 70 per cent. The standard curves for all preparations were essentially parallel over the entire range.

## 1 THE UNIT OF HYALURONIDASE

Different batches of hyaluronate vary in purity and degree of polymerization and therefore may produce different turbidities at a given concentration. For assay purposes, these variations can be eliminated by using all substrate preparations at a standard turbidity rather than on a constant weight basis as originally suggested by Kass and Serstone.<sup>4</sup>

The hydrolysis of hyaluronate proceeds as a monomolecular type reaction expressed by the formula  $k = \frac{1}{t} \log \frac{a}{(a-x)}$ .<sup>5</sup> This relationship is true within those limits of substrate concentration which give a straight line standard curve. The assay of hyaluronidase can be carried out on this portion of the curve by choosing an initial turbidity of  $50 \pm 5$  per cent light transmission or 200 to 250 turbidity readings on the Klett instrument. Under the conditions of our assay method as described below it is therefore possible to establish a unit for measuring activity of the enzyme. One unit of hyaluronidase is defined as the amount which will hydrolyze one half of a quantity of substrate sufficient to cause a turbidity corresponding to 50 - 5 per cent light transmission.

## 2 ASSAY METHOD FOR HYALURONIDASE

1 *Preparation of Stock Solutions*—(a) Two buffers are used both made from stock solutions of 0.5M sodium acetate and 0.5M acetic acid. One tenth N acetate buffer pH 6.0 is made by combining 3 ml of 0.5M acetic acid, 97 ml of 0.5M sodium acetate and 4.384 Gm of sodium chloride and diluting to 500 ml with water. This gives a concentration of 0.15M sodium chloride. Half N acetate buffer pH 4.2 is made by combining 130 ml of sodium acetate with 370 ml of 0.5M acetic acid. The pH of the buffers should frequently be checked and adjusted if necessary.

(b) Potassium hyaluronate is dissolved in the 0.1M acetate sodium chloride buffer, pH 6.0. On the basis of the standard curve a concentration is chosen which in the assay will give a transmission of  $50 \pm 5$  per cent at a wave length of 600 m $\mu$  which corresponds to a turbidity reading of 200 to 250 in the Summer son Klett photoelectric colorimeter using a blue filter. For many preparations of hyaluronate, this turbidity will be reached by using a concentration of 0.4 mg per 1.0 ml of water. Otherwise, the concentration of hyaluronate should be adjusted to give a turbidity reading of about 225. The solution is stored at 5° C and is warmed to room temperature before use. It can remain at 5° C without changing, over a period of at least two weeks.

(c) Hyaluronidase is dissolved at room temperature, in the pH 6.0 acetate sodium chloride buffer. The concentration is chosen so that 1 ml contains approximately 3 units. The enzyme solution is used immediately after being made.

<sup>5</sup> In this formula  $k$  = velocity constant  $t$  = time  $a$  = initial concentration of substrate  $x$  = quantity of substrate hydrolyzed during time  $t$ .

(d) Acidified protein solution is prepared by diluting human plasma 1 to 10 with 0.5M acetate buffer, pH 4.2 and adjusting to pH 3.10 with 4N hydrochloric acid. This solution is distributed in 50 cc portions in test tubes measuring 25 by 200 milliliters. These tubes are placed in a boiling water bath. After the plasma reaches temperature equilibrium ( $99^{\circ}\text{C}$ ), it is kept in the bath for thirty minutes, when it is immediately cooled to room temperature by immersion in ice water. The plasma can be kept in the cold for at least two weeks.

2 *Incubation of Enzyme and Substrate*—Seven Klett tubes are set up for each assay as shown in Table IV. The solutions are mixed and the tubes are

TABLE IV SETUP OF TUBES FOR HYALURONIDASE ASSAY

KLETT TUBE	SUBSTRATE SOL	pH 6 BUFFER	ENZYME SOL
#1	5 ml	5 ml	—
2	25	75	—
3	5	—	5 ml
4	5	1	4
5	5	2	3
6	5	3	2
7	5	4	1

immersed in a constant temperature water bath kept at  $37.5^{\circ}\text{C}$  for thirty minutes. In order to inactivate the enzyme, the tubes are immersed in a  $60^{\circ}\text{C}$  water bath for ten minutes. They are cooled to room temperature under running water. As a rule, not more than three enzyme fractions and the blanks are assayed simultaneously (seventeen tubes). If more tests have to be performed, they are staggered in order to keep the time relations constant.

3 *Development of Turbidities*—To each Klett tube are added 3 ml of 0.5M acetate buffer, pH 4.2, followed by 1 ml of acidified human plasma treated as described. The contents of each tube are mixed and then allowed to stand at room temperature for thirty minutes. The turbidities are read in the Summerson-Klett photoelectric colorimeter with a blue filter or any other suitable instrument. The zero reading of the instrument is set with a tube containing 1 ml of pH 6 acetate sodium chloride buffer, 3 ml of pH 4.2 acetate buffer and 1 ml of acidified, stabilized human plasma solution.

All Klett tubes are numbered permanently and their absorption which is variable is determined with the same mixture which is used to set the zero of the instrument. For the zero reading, only tubes with zero absorption are used. The readings of the seventeen tubes in the assay are corrected to zero absorption.

4 *Method of Calculation*—The corrected turbidity readings are plotted against concentration of enzyme. This gives a straight-line curve since the Klett instrument has a logarithmic calibration. The enzyme concentration corresponding to the turbidity of the lower blank is read from the graph. This indicates the amount of enzyme containing 1 turbidity reducing unit.

#### G THE ACCURACY OF THE ASSAY

In order to determine the error in this procedure, seventy-one assays were made on thirty-two different days by two different technicians, using the same

sample of enzyme. The mean value of all determinations was 27 TRU per milligram. The results were treated statistically and the standard deviation of a single observation is 1.0. The distribution of the assay results is shown in Fig. 6.

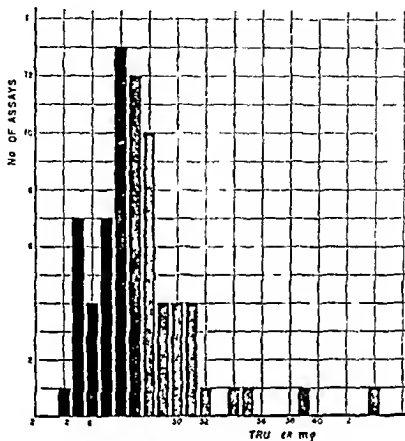


Fig. 6—Distribution of seventy one assay results

#### CONCLUSIONS

The assay method of Kass and Seastone<sup>4</sup> has been used with modifications by Leonard and associates and Meyer. Recently Dorfman and Ott<sup>5</sup> have published a critical investigation of various phases of the turbidimetric technique. Having used the turbidimetric method for several years in this laboratory, we find it important to emphasize the following points in order to clarify some of the discrepancies in the results obtained by different laboratories.

The enzymatic hydrolysis of hyaluronic acid is greatly influenced by the nature and concentration of the buffers employed. We find that a suitable buffer is 0.1M sodium acetate pH 6.0, containing 0.15M sodium chloride. This is close enough to the optimum salt concentration for hyaluronidase activity and measures the enzymatic activity under physiologic conditions.

Since the accuracy of the method is dependent upon the length of time of hydrolysis, it is expedient, in performing large numbers of routine assays to inactivate the enzyme after the thirty minute incubation period. This can readily be achieved by immersing the reaction tubes in a water bath of 60° C for ten minutes.

The turbidimetric method of measuring the amount of hyaluronic acid remaining after enzymatic hydrolysis is based on the interaction between hyaluronic acid and acidified protein. It has been mentioned by Seastone<sup>4</sup> and by



Leonard and co-workers<sup>5</sup> that the age of the protein solution influences the development of turbidities. As we have shown, aging by rigidly standardized heating will insure reproducible and accurate results. The protein is denatured during the stabilizing process and as a result becomes more precipitable. This results in a greater turbidity when the protein reacts with hyaluronic acid. The slopes of the standard curves obtained with increasing amounts of hyaluronic acid and stabilized protein are much steeper than those obtained with unheated protein. This increases the difference in the turbidity of the two blanks and the accuracy of the assay. It is also important to age the proteins at the proper pH in order to obtain maximum turbidities. Under the conditions of our experiments, aging should be done at pH 3.1.

In the present study, all assays have been carried out with hyaluronate prepared from umbilical cords. Different batches of substrate vary somewhat in their chemical and physicochemical properties and give different turbidities with acidified protein. For assay purposes, these variations can be eliminated by adjusting all substrate solutions to a standard turbidity.

By utilizing the information collected during this study it has been possible to arrive at a method for the assay of hyaluronidase which is accurate, reproducible, and permits the definition of a unit without reference to a standard.

#### SUMMARY

1. Irregularities in the turbidimetric assay of hyaluronidase have been demonstrated and traced to the protein employed in forming the turbidity with the substrate hyaluronic acid.

2. Various proteins have been investigated and methods devised for their stabilization by heating under specified conditions.

3. Other factors influencing the accuracy of the method have been studied.

4. A study of the substrate has been made and methods of preparation and use have been described which give constant assay results from batch to batch.

5. An assay method for hyaluronidase using heat-stabilized human plasma is described.

6. A unit for hyaluronidase activity has been defined and discussed.

The authors wish to thank Anne H. Kennison and James W. Cassidy for their assistance in this investigation.

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# SICKLE CELL DISEASE STUDIED BY MEASURING THE SURVIVAL OF TRANSFUSED RED BLOOD CELLS

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THE investigations to be described in this report were designed (1) to study the pathogenesis of the increased hemolysis in patients with sickle cell anemia, (2) to determine how sickle cell anemia and sickle cell trait erythrocytes differ in terms of survival time following transfusion, and (3) to search for evidence that forms of sickle cell disease intermediate between the anemia and the trait might exist. Survival of transfused red blood cells was measured with the Ashby technique of differential agglutination<sup>1</sup>. Observations were made (1) on the survival of normal cells transfused into patients with sickle cell anemia, (2) on the survival of cells transfused from donors with the sickle cell trait and from donors with sickle cell anemia, (3) on the effect of high tensions of inspired oxygen on the survival of cells transfused from patients with sickle cell anemia, and (4) on the survival of sickle cell trait cells transfused into a subject with chronic cyanosis. The results clearly indicate that the abnormal hemolysis of sickle cell anemia is caused by an intracellular defect which makes the cells less able to stand the trauma of circulation. Trait erythrocytes, on the other hand, survive normally.

## METHODS AND EXPERIMENTAL CONDITIONS

The modified Ashby technique of Dicke and Molison<sup>2</sup> was used for determining the nonagglutinable counts. Differentiation of transfused cells was made by either the ABO groups or the MN groups†. Counts were made from venous blood at frequent intervals following transfusion, usually by two independent observers. Each observer took the average of at least four counts. In earlier experiments a crude measure of survival of transfused sickled cells in normal subjects was made by counting the percentage of sickle cells in sealed wet preparations of finger blood after forty eight hours. Later, to insure full sickling, CO was bubbled for twenty minutes through samples of venous blood kept under paraffin oil, the cells were then fixed with 10 per cent formalin in 0.85 per cent sodium chloride. Moist preparations were made from this blood and the number of abnormal forms found in 100 cells was counted. The absolute number of sickle cells was calculated from this figure and the total red cell count. Red blood cell pipettes and hemocytometer chambers were standardized by the United States Bureau of Standards. Wet preparations were used in Sherman's test<sup>3</sup> for the percentage of sickled cells in venous blood.

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TABLE I. HISTOLOGIC DATA AND CHARACTERISTICS OF SURVIVAL OF NORMAL RED BLOOD CELLS TRANSFUSED INTO PATIENTS WITH SICKLE CELL ANEMIA, AND OF SICKLE TRAIT CELLS GIVEN TO NORMAL SUBJECTS A PATIENT WITH SICKLE CELL ANEMIA AND ONE WITH CHRONIC CYANOSIS. RESULTS OF INFUSIONS OBSERVATIONS ON NORMAL RECIPIENT SUBJECTS AND SUBJECTS WITH HEMOLYTIC ANEMIA TRANSFUSED WITH NORMAL BLOOD ARE SHOWN FOR COMPARISON.

DONOR				RECIPIENT				CHARACTERISTICS OF SURVIVAL												
NAME	SEX	AGE	DIAGNOSIS	HB (GM/100 CC)	RBC (MILLIONS/MM <sup>3</sup> )	RETIC (%)	S* (%)	NAMP	SPC	AGE	DIAGNOSIS	HB (GM/100 CC)	RBC (MILLIONS/MM <sup>3</sup> )	RETIC (%)	S (%)	L	I	z	r	F
4 normal female subjects																				
4 normal male subjects																				
6 subjects with hypochromic anemia																				
T J	F	15	Sickle cell anemia	63	22										39.8	0.0084	0.0001	0.7	116	0.01
B W	F	20	Sickle cell anemia	00	278										37.3	0.0074	0.0011	0.9	102	0
H S	F	14	Sickle cell anemia	76	28										43.9	0.0094	0.0116	0.3	112	0.36
A Th	F	0	Hysterectomy	116	312										0	0.0081	(+)	0.2	124	(+)
A Tu	M	19	Normal	117	501										0	0.0091	0	0.5	110	0
K H	F	2	Fracture	130	41										0	0.0080	0.002	0.7	126	0.14
R F	M	40	Fracture	119	19										0	0.011	(0)	0.3	86	(0)
H So	F	64	Osteoarthritis	132	402										0	0.0082	(0)	0.1	120	(0)
H S	F	14	Sickle cell anemia	79	269										++	0.0093	0.0041	0.1	108	0.22
H H	M	14	Etiology complex	224	732										0	0.0077	0	0.6	130	0

Abnormal and sickle cells, Sherman test.

\*Results from Callender and co workers<sup>1</sup> and Brown and co workers<sup>2</sup> and some unpublished data.

†Counts erratic. Curvature appreciable but not significant.

‡ (the average time of survival of transfused cells) and r (the end point of the curve) are in days from transfusion.

Other symbols see text.

Normal cells for transfusion were obtained from the Blood Bank of the Barnes Hospital. White donors were selected to insure absence of sickling. The blood was less than five days' old when used. Donors with sickle cell anemia or the sickle cell trait were bled by one of us as needed for the experiments, and the cells were transfused within twenty-four hours. Acid citrate dextrose (A.C.D.) solution was used as the preservative and anticoagulant, supernatant plasma was removed immediately before transfusion.

All of the patients with sickle cell anemia showed the characteristic clinical manifestations of the disease, in anemia, reticulocytosis, elevated icteric index, more than 30 per cent abnormal or sickled cells with Sherman's test, and all have required repeated transfusions for support.

### SURVIVAL OF NORMAL CELLS TRANSFUSED TO A PATIENT WITH SICKLE CELL ANEMIA

RECIPIENT T J, ♀, COL AGE 25 GROUP B, RH NEG  
HG 6.3 GM, RBC 2,200,000/mm<sup>3</sup>,  
RETICS 22/  
SHERMAN TEST 398/ ABNORMAL AND SICKLE CELLS

DONORS NORMAL WHITE DONORS, O, RH NEG

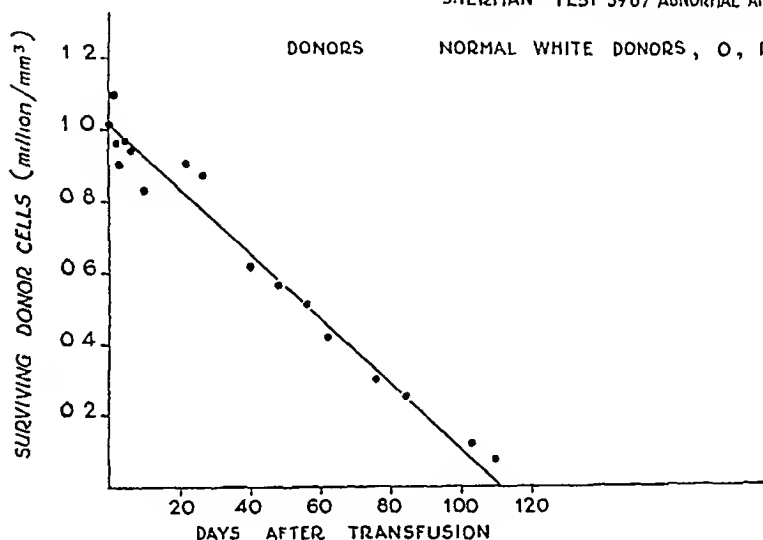


Fig 1

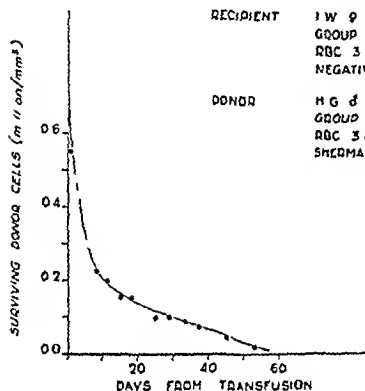
"Normal" recipient subjects were patients in the hospital who were either hematologically normal, in which case an equivalent amount of blood was removed immediately prior to the transfusion, or they were women with a moderate hypochromic or normocytic anemia who had recently had a hysterectomy for menorrhagia. They were all negative for the sickling trait.

In the experiments involving continuous inhalation of oxygen, 70 to 80 per cent oxygen was given by an O.E.M. mask. An oronasal mask was used except at mealtimes when a nasal mask was substituted. The flow was kept at 7 to 8 liters per minute and a humidifier was used. A nurse was in constant attendance at the bedside to keep the mask properly adjusted.

### RESULTS

I *The Survival of Normal Cells Transfused Into Patients With Sickle Cell Anemia*—Normal red blood cells were transfused into three different patients with sickle cell anemia. The results (Fig 1, Table I) are in agreement with those of Altmann<sup>4</sup> in that the survival times of the transfused cells were normal.

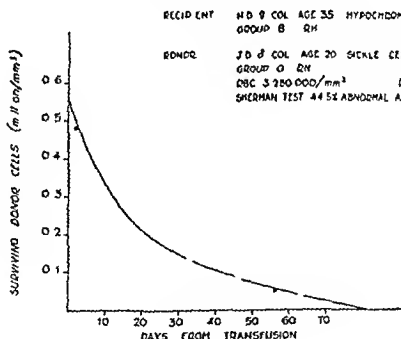
# SURVIVAL OF CELLS TRANSFUSED FROM A PATIENT WITH SICKLE CELL ANEMIA



RECIPIENT 1 W 9 COL AGED 37 HYPOCHROMIC ANEMIA  
 GROUP A RH+ HB 6.3 GM  
 RBC 3 170 000/mm<sup>3</sup> RETICS 2%  
 NEGATIVE FOR SICKLING TRAIT  
 DONOR HG 8 COL AGED 23 SICKLE CELL ANEMIA  
 GROUP O RH+ HB 9.84 GM  
 RBC 3 510 000/mm<sup>3</sup> RETICS 11.5%  
 SHERMAN TEST 44% ABNORMAL AND SICKLE CELLS

FIG

# SURVIVAL OF CELLS TRANSFUSED FROM A DONOR WITH SICKLE CELL ANEMIA



RECIPIENT HD 9 COL AGE 35 HYPOCHROMIC ANEMIA  
 GROUP B RH  
 DONOR JD 8 COL AGE 20 SICKLE CELL ANEMIA  
 GROUP O RH HB 11.0 GM  
 RBC 3 280 000/mm<sup>3</sup> RETICS 50%  
 SHERMAN TEST 44.5% ABNORMAL AND SICKLE CELLS

FIG 3

II *The Survival of Red Cells Transfused From Patients With Sickle Cell Anemia*—Very different results were obtained when the positions of donor and recipient were reversed (Figs 2 and 3). Four normal subjects received blood from patients with sickle cell anemia. In each instance there was a rapid destruction of the majority of the transfused cells. The graphs of survival, however, differed from those found in conditions where there is indiscriminate

destruction of red cells, e g in patients with acquired hemolytic anemia transfused with normal cells<sup>7</sup> There was a rapid initial fall but the speed of hemolysis was not maintained and a small proportion of the sickle cells, varying from case to case, tended to survive for a longer period

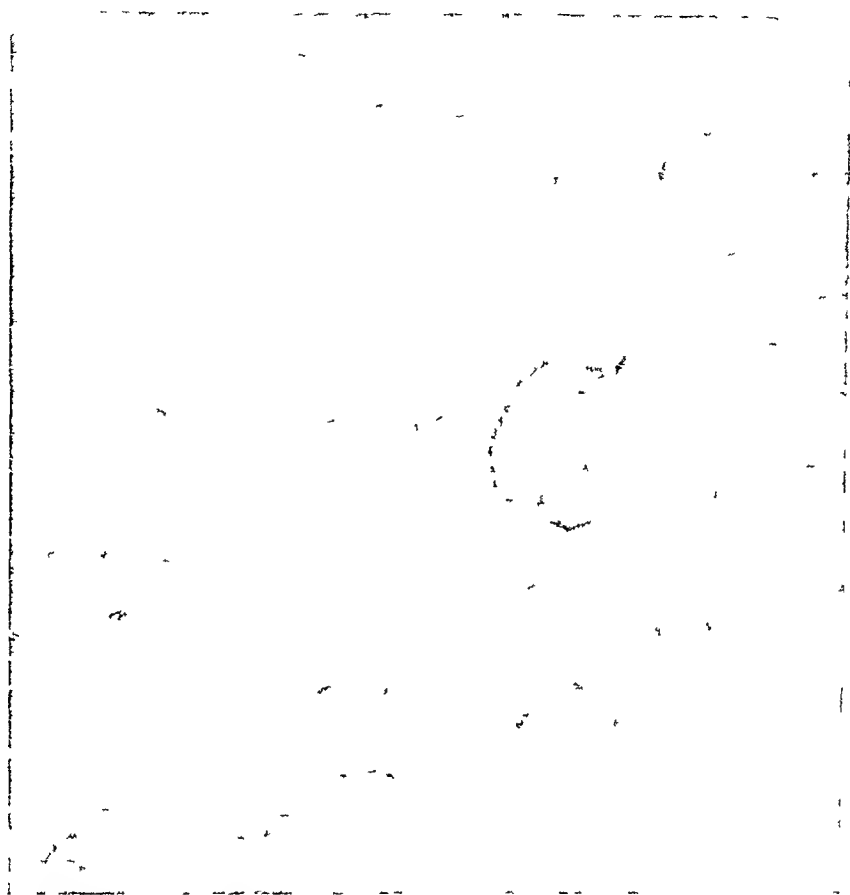


Fig 4—Unstained wet preparation of blood of normal recipient (A Tu) showing sickle cells transfused from a patient with sickle cell anemia (B W) about two weeks previously

Survival of the sickle cells was also studied by counts made on sealed moist preparations of finger blood This method did not insure sickling of all potential sickle cells and, therefore, did not give a true measure of survival of the transfused cells Sickling could, however, be demonstrated for about three weeks from transfusion (Fig 4)

One patient (G P), with sickle cell anemia, received blood from another girl (L B) with sickle cell anemia The disappearance of the transfused sickle cells was comparable to that found in the normal recipient subjects, again there was a suggestion of some selective destruction (Fig 5)

III *The Survival of Red Cells Transfused From Donors With the Sickle Cell Trait*—When five healthy, Negro subjects having the sickle cell trait were used as donors, the results were in striking contrast to those obtained with blood

## SURVIVAL OF SICKLE CELLS TRANSFUSED TO A PATIENT WITH SICKLE CELL ANEMIA

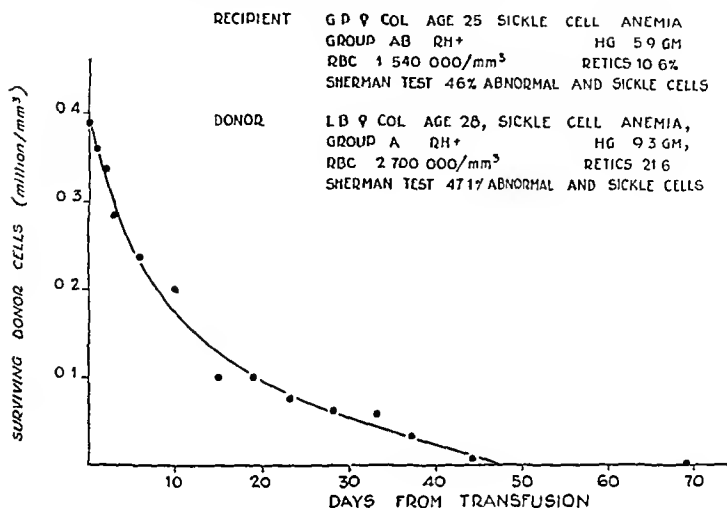


Fig 5

## SURVIVAL OF CELLS TRANSFUSED FROM A DONOR WITH SICKLE CELL TRAIT

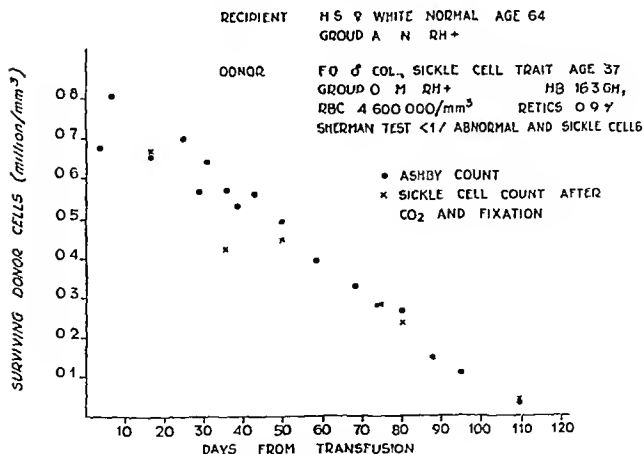


Fig 6



transfused from patients with sickle cell anemia (Table I) Four of five normal recipients showed normal survival of the transfused trait cells Fig 6 illustrates this result and shows also the close correlation between the nonagglutinable counts obtained by the Ashby technique and the number of surviving cells calculated from sickle cell counts made on venous blood samples treated with  $\text{CO}_2$  The cells from the fifth donor (A H) showed a slightly shorter survival time The recipient in this instance did not report regularly for observations and the data are not entirely satisfactory

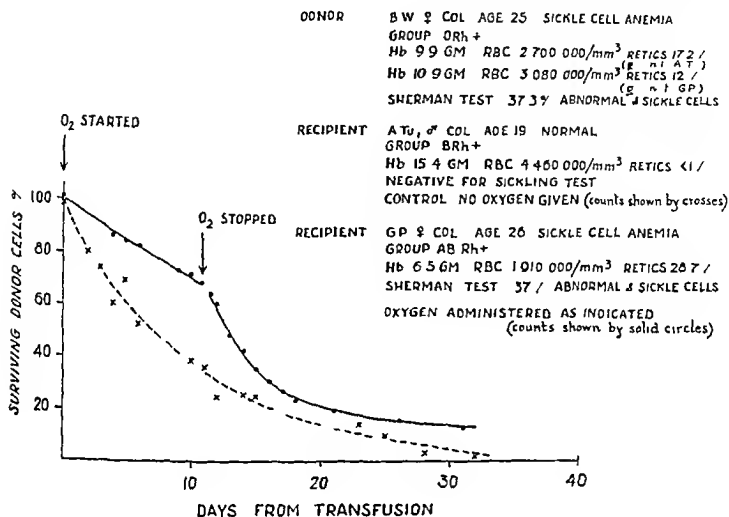
One of the donors (F O) with sickle cell trait gave blood on a second occasion for transfusion to the girl (H S<sub>1</sub>) with sickle cell anemia, previously transfused with normal cells The two graphs of survival in this girl were strikingly similar

IV *The Effect of High Tensions of Inspired Oxygen on the Survival of Cells Transfused From Subjects With Sickle Cell Anemia*—The patient G P (vide supra) with sickle cell anemia was given a second transfusion with cells from another donor with sickle cell anemia (B W) This time inhalation of oxygen was started about two hours before transfusion and continued for ten days Eighty per cent oxygen was used initially but this was reduced to 70 per cent on the second day The results are shown in Fig 7 Although destruction of transfused cells was abnormally rapid during the first ten days, it was slower than on the occasion of the previous transfusion When oxygen was discontinued an acceleration in the cell destruction was indicated by an increase in the slope of the graph The initial slope was about  $0.029 \text{ day}^{-1}$ , after withdrawal of oxygen,  $0.112$  The difference is considerable, and the second figure compares well with the initial slope in another recipient (A Tu) receiving blood from the same donor (0.116)\* Sickle counts made on venous blood samples at intervals by Sherman's technique<sup>3</sup> showed a reduction in percentage of total abnormal plus sickle cells from 36.8 to 10.8 during oxygen inhalation and a corresponding reduction of true sickle cells from 22 to 0.8 per cent As observed previously by Reinhard and co-workers,<sup>8</sup> the percentage of abnormal and sickle cells showed a rise after oxygen inhalation was stopped, in this instance, the abnormal cells increased to 50.8 per cent and completely sickled cells to 39.0 per cent before returning to base line figures

Two similar experiments were planned using normal recipient subjects The first of these (M L) tolerated oxygen somewhat irregularly for only two days During this time the nonagglutinable count had shown little change, whereas subsequently there was a rapid disappearance of transfused cells This cannot, however, be taken as a reliable experiment The second normal recipient subject in this group (Z R) cooperated well She inhaled 80 per cent oxygen for twenty-four hours after transfusion and then 70 per cent for an additional

\*Sometimes the inagglutinable counts are much more erratic than they are here the configuration of the data might possibly have arisen from an unfortunate conjunction of random error The following argument may therefore be made If to the data are fitted any of the survival functions that have been proposed under the hypothesis that the oxygen has had no effect it is found on applying Jeffreys' K test<sup>9</sup> that even for the 4-parameter equations K is less than 0.1 Thus it is highly improbable that the divergence of the data in question from the fitted curves is due to random and not systematic error Further it may be noted that the chance of the observed change in slope occurring just where it does between say ten and thirteen days and not elsewhere is of the order of 0.1 if it is merely accidental

## EFFECT OF INHALATION OF 70% OXYGEN ON THE SURVIVAL OF TRANSFUSED SICKLE CELLS



Fig

eight days. There was no significant change in slope of the decay curve on withdrawing oxygen. But this does not necessarily mean that the oxygen had no effect. The slope of the first section was considerably less than might have been expected from a previous experiment with the same donor (0.043 day<sup>-1</sup> as against 0.058).

V *The Survival of Sickle Trait Cells in a Subject With Chronic Cyanosis*—An attempt to accelerate the disappearance of cells transfused from a donor with the sickle cell trait was made by giving a replacement transfusion to a boy with congenital heart disease (Eisenmenger's complex) and extreme cyanosis (Table I). The arterial oxygen saturation was 80.5 per cent as compared with a normal control of 95.5 per cent\*. The survival time of the transfused cells did not differ from that found in normal recipient subjects.

VI *Mathematical Analysis of Results*—Transfusions of normal and sickle trait blood into subjects with sickle cell anemia and of sickle trait blood into normal subjects have in each case led to a decay curve of a linear or gently and uniformly curved character having, with one doubtful exception, a rather well defined end point near 120 days. Curves of this type are such as have been associated with transfusions of normal blood into normal subjects, and to make comparison quantitative, an equation of one of two forms has been fitted to

\*The authors are indebted to Dr John C. Tinsley, Jr. for these determinations.



TABLE II. HEMATOLOGIC DATA AND CHARACTERISTICS OF SURVIVOR OF RED BLOOD CELLS TRANSFUSED FROM DONORS WITH SICKLE CELL ANEMIA

NAME	SEX	AGE	DONOR					RECIPIENT					CHARACTERISTICS OF SURVIVAL					
			DIAGNOSIS	Hb (gm/100 cc)	PBC (millions/mm <sup>3</sup> )	PTIC (%)	S (%)	TRANSFUSION	AGE	DIAGNOSIS	Hb (gm/100 cc)	HbC (millions/mm <sup>3</sup> )	PTIC (%)	S (%)	$\alpha$	$-\beta$	$\gamma$	$\pi$
B W	F	25	Sickle cell anemia	9.9	275	17.2	37.3	M	10	Normal	1.1	1.16	<10	0	0.10	0.00	0.1	0.05
J D	M	22	Sickle cell anemia	11.0	33	3.0	40.6	D	2	Iron deficiency anemia	1	1	<10	0	0.13	0.00	0.1	0.05
H G	M	23	Sickle cell anemia	9.8	315	11.5	44.0	W	1	Iron deficiency anemia	1.3	1.1	30	0	0.0	0.00	0.48	0.0
L B	F	30	Sickle cell anemia	9.3	2	21.6	47.1	L	20	Sickle cell anemia	1	1.4	10	10	0.13	0.00	0.11	0.0
B W	F	25	Sickle cell anemia	10.9	32	12.0	44	P	20	Sickle cell anemia	1.1	1.91	30	30	0.13	0.00	0.11	0.0
J D	M	22	Sickle cell anemia	11.9	41	10.0	33.6	R	1	Normal	1.0	1.5	0.4	0				
W G	M	23	Sickle cell anemia	7.0	224	19.6	41.2	M L	21	Sickle cell anemia	1.1	1.01	0.6	0				

\*Abnormal and sickle cells Sherman test

† 7 in days

Other symbols see text

Oxygen inhaled for a period following transfusion

The evidence suggests that the red cells' environment is normal in sickle cell anemia, and so the transfused cells should behave as they would have done if they had remained in the donor's circulation. This is further supported by an experiment in which both donor and recipient were suffering from the anemia (L B to G P, Table II). The relation given by Callender and associates<sup>5</sup> between the survival of transfused cells and the life span as measured from "birth" may, therefore, be applied. If the chance that a cell will live for a time equal to or greater than  $\tau$  is  $\varphi(\tau)$ , then

$$\varphi(\tau) = - \frac{N_1}{n} \left\{ \frac{d}{dt} \psi(t) \right\} \Big|_{t = \tau} \quad \text{--- --- --- --- --- III}$$

where  $\psi(t) = N/N_0$  so that  $\psi(0) = 1$ ,  $N_1$  is the donor's red cell count,  $n$  is the rate of production of his cells per unit volume of blood, and  $N_1/n$  is their true average life. This involves, *inter alia*, the dangerous assumption that  $n$  is reasonably constant for some time before the blood is drawn, but one cannot proceed

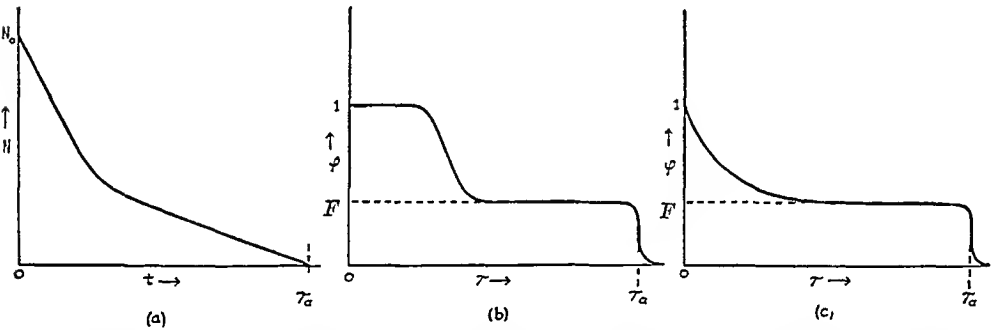


Fig 8—(a) An idealised form of the survival curve of transfused sickle cells. (b) and (c) Possible forms of  $\varphi(\tau)$  the chance that a cell will live for a time  $\tau$  from its release into the blood stream

without it, and it has some support from the great similarity of all the survival curves in this group, if  $n$  has been widely variable, the similarity would have been a most improbable occurrence. Equation III asserts that, apart from a constant factor,  $\varphi(\tau)$  is the derived curve of  $\psi(t)$ . So if  $\psi(t)$  is of the form shown in Fig 8,a (which is an idealised and sharpened version of the sickle cell survival curve) then  $\varphi(\tau)$  is as shown in Fig 8,b. Actually, however, because of its high slope and because the first few counts are usually erratic, the first section of the curve is so poorly determined that  $\varphi(\tau)$  might equally be of the form of Fig 8,c. We shall in fact assume that the latter form holds, it may be written as the sum of a constant and a variable term. The latter we take for convenience to be exponential, and write

$$\left. \begin{aligned} \varphi(\tau) &= F + (1 - F) \exp(-L_e \tau), & \tau < \tau_a \\ &= 0, & \tau > \tau_a \end{aligned} \right\} \quad \text{--- --- --- --- --- IV}$$

where  $\exp(-L_e \tau)$  is to be understood as a gross approximation to some function, initially 1, which falls to a small value in a time of the order of  $1/L_e$ .  $\varphi(\tau)$  then

contains three parameters,  $F$ ,  $L_e$  and  $\tau_a$ , and so  $N$  which is an integral of  $\varphi(\tau)$ , contains four, a hyperbolic  $\psi$  like Fig 8,a would require five in  $N$

It is important to distinguish the situation here represented by equation IV from the one in which the cells are all equally subject to a nonselective random destructive process as well as a process of certain destruction if they reach the age  $\tau_a$ . For then

$$\varphi(\tau) = \exp(-L_e \tau) \quad \tau < \tau_a \quad \left. \begin{array}{l} \\ = 0, \quad \tau > \tau_a \end{array} \right\}$$

$$\text{and hence } \psi(t) = N/N_0 = \frac{\exp(-L_e t) - \exp(-L_e \tau_a)}{1 - \exp(-L_e \tau_a)} \quad \text{--- V}$$

(This is the exact form of equation II)

$$\frac{d\psi}{dt} = \psi'(t) = - \frac{L_e \exp(-L_e t)}{1 - \exp(-L_e \tau_a)}$$

Now if  $L_e$  is large,  $\psi'(0)$  is large and  $\exp(-L_e \tau_a)$  is very small as they are in transfusions from patients with sickle cell anemia but the  $\psi(t)$  of equation V approaches 0 closely long before  $t = \tau_a$  and  $\psi'(\tau_a)$  is also very small. Therefore the uniform slope of the last portion of the sickle cell anemia survival curve cannot be accounted for by the operation of a highly active, constant, nonselective hemolysis.

We return then to equation IV. It admits of two simple interpretations.

(a) All the red cells have a potential life span  $\tau_a$ . This is less than the normal value and differs from case to case. But in addition they have some inherent deficiency which is gradually corrected as their age increases, many are as a consequence destroyed at an early age, but some survive to attain the age  $\tau_a$ .  $F$  is the fraction which do so survive and  $L_e$  is a measure of the rate of destruction of those which do not.

(b) The red cells leaving the marrow of the donor are of variable quality. A fraction  $F$  live for the same time  $\tau_a$  and after transfusion give rise to the linear tail of the survival curve,  $1 - F$  are more labile and are more or less rapidly destroyed in a time whose average value is of the order of  $1/L_e$ .

The second of these interpretations is the more likely, especially as the red cell cannot be regarded as a living organism. The existence of a small component of random cell destruction, normal in women, has here been neglected. The data are in any case insufficient to allow of its separate estimation.

In order to obtain an equation suitable for fitting to the survival data of sickle cell anemia cells, we integrate IV and find

$$N = \alpha + \beta t + \gamma \exp(-L_e t) \quad \text{--- VI}$$

where approximately

$$\begin{aligned} \alpha &= N_0 \frac{F}{F + (1 - F)/L_e \tau_a} \\ \beta &= N_0 \frac{F}{F + (1 - F)/L_e \tau_a} \\ \gamma &= N_0 \frac{(1 - F)/L_e \tau_a}{F + (1 - F)/L_e \tau_a} \\ \alpha + \gamma &= N_0 \end{aligned}$$

(In these expressions terms containing the factor  $\exp(-L_c\tau_a)$  have been suppressed since in our application  $L_c\tau_a$  is never less than 5 and  $\exp(-5) = 0.007$  only) The results of the fitting are given in Table II. The goodness of fit is satisfactory, and from the values of  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $L_c$  have been calculated the same three quantities as before

The average time of survival of the transfused cells,  $\bar{t}$

$$\bar{t} = \frac{\gamma/L_c - \alpha^2/2\beta}{\alpha + \gamma}$$

The end point of the curve,  $\tau_a$

$$\tau_a = -\alpha/\beta$$

The proportion of transfused cells of abnormally short life, those we have represented as destroyed by an exponential hemolytic process,  $F_c$

$$F_c = \gamma/(\alpha + \gamma)$$

$F_c$  is also the proportion of cells of the short-lived type in the donor's circulation,  $1 - F_c$  the proportion surviving to age  $\tau_a$

In this short series no well-defined correlation of survival time with the blood picture of the donors is evident

There is however an interesting regularity in the values of  $F_c$ , they range only from 0.65 to 0.70, and so to the present degree of accuracy  $F_c$  can be regarded as constant. With so few examples to draw from it would be unsafe to read any physiologic significance into this, rather it suggests that only two and not three variable parameters are necessary in the expression of  $\varphi$ , three and not four in  $N$ . Equations IV and VI are thus perhaps unnecessarily complex, but we have so far no real guidance as to how they should be modified.

#### DISCUSSION

These results indicate clearly that the increased hemolysis in sickle cell anemia is a function of the abnormal erythrocyte itself, there is no evidence that hormonal or erythrophagic factors are directly responsible. Similar conclusions have been reached by Singer, King, Robin, and Jefferson in independently conducted studies<sup>10</sup>. It seems likely that the abnormal shape of the sickled cell makes it less able to tolerate the trauma of circulation. Compatible with this explanation is the *in vitro* demonstration by Diggs and Bibb<sup>11</sup> that cells from patients with sickle cell anemia, when shaken in an atmosphere of carbon dioxide, hemolyse more readily than do normal cells so treated. Shen, Castle, and Fleming<sup>12</sup> obtained similar results with blood from one patient with sickle cell disease. It is not entirely clear whether this patient had the trait only or the active disease, presumably the former as the hematocrit was 44 per cent.

One of us (J. N.),<sup>13</sup> in an extensive study of the susceptibility of red cells to mechanical trauma, has repeatedly confirmed this finding of decreased resistance of sickled cells, and has shown, furthermore, that such cells are normally resistant when fully oxygenated and in the form of biconcave discs.

One would expect, therefore, that if intravascular sickling could be reduced, the rate at which cells from patients with sickle cell anemia are destroyed would

be decreased. The earlier observations of Reinhard and co workers<sup>8</sup> showing with the Sherman technique that intravascular sickling is decreased when patients breathe 70 to 80 per cent oxygen were again confirmed in the experiments described herein. It is not surprising, therefore, that erythrocytes transfused from a patient with sickle cell anemia seemed to survive longer in one of the subjects who breathed high concentrations of oxygen. We are unable to correlate this observation with the fact that intravascular hemolysis as measured by total urobilinogen excretion did not seem to be decreased in patients with sickle cell anemia during periods of oxygen inhalation.<sup>9</sup> The urobilinogen methods may not have been sensitive enough to detect the relatively slight decrease in rate of destruction.

Previous workers have suggested that ease of sickling is a function of the age of the cells because they observed that nucleated red cells and reticulocytes appear to sickle less readily than do other erythrocytes.<sup>11, 14, 15</sup> However, if sickling is mainly a function of age the life expectancy of all cells in sickle cell anemia should be reduced. Instead the graphs of survival of the transfused sickle cell anemia erythrocytes show that there is a tendency for a proportion of the cells to survive almost normally.

Case 3 of Singer and associates<sup>10</sup> in which blood from two donors with sickle cell anemia was given to one subject with sickle cell trait shows a similar type of graph. This was interpreted as being due to admixture of blood from a severe and a milder form of the anemia. Our data suggest an alternative interpretation namely that while most of the cells are constitutionally more susceptible to sickling and remain so until they are prematurely destroyed others are born more resistant and remain so throughout their existence. According to this hypothesis sickle cell trait might be regarded as the extreme example of a state in which all the cells are constitutionally more resistant to sickling.

There are other reasons for thinking that the difference between sickle cell anemia and the trait is qualitative. (1) In patients with the trait there are fewer circulating sickled forms<sup>3</sup> even though most of the cells must be older than in sickle cell anemia. (2) Transfused trait cells survive normally in spite of their average greater age and their ability to sickle. (3) When trait cells are completely sickled by exposure to CO and then subjected to mechanical trauma they are much less readily destroyed than are anemic cells under similar circumstances.<sup>13</sup> It is difficult to interpret the experiment in which no accelerated destruction of trait cells occurred when they were transfused into a young boy with chronic cyanosis from congenital heart disease. That result may be another indication of the resistance of trait cells to sickling and to abnormal hemolysis. On the other hand the oxygen tension may have to be lowered to 18 mm mercury<sup>3</sup> before sickling occurs and it may well be that such low tensions were never reached in this boy's circulation in spite of his cyanosis.

If the difference between the two types of sickle cell disease is caused by a qualitative difference in the erythrocyte structure, it is easier to understand why no entirely satisfactory, clear cut examples of forms intermediate between the two have been described. In these studies a search was made for evidence that there might be a continuous spectrum in the behaviour of cells transfused from subjects with sickle cell disease ranging from the severe anemia through milder



forms to the trait With the one exception provided by an unsatisfactory experiment (subject A H, Table I), however, there was a sharp distinction between the survival graphs of cells transfused from patients with the anemia and those with the trait

#### SUMMARY AND CONCLUSIONS

The Ashby technique of differential agglutination has been used to study the survival of transfused cells in relation to sickle cell disease

Normal red blood cells transfused to three patients with sickle cell anemia showed a normal survival time

Red cells from patients with sickle cell anemia transfused to normal recipient subjects and others with sickle cell anemia showed a shortened average time of survival

Red cells from healthy donors with the sickle cell trait transfused to normal recipient subjects and a patient with sickle cell anemia showed a normal survival time

Oxygen inhalation reduced the rate of destruction of transfused sickle cell anemia cells in one patient with sickle cell anemia Such an effect was not seen in one normal recipient The survival time of sickle trait cells given to a boy with chronic cyanosis was normal

The findings indicate that the defect in sickle cell anemia is inherent in the red blood cell There is evidence to suggest that sickling is not, as has been thought, a function of age of the cell but that the cells in sickle cell anemia vary constitutionally in their liability to sickle It is suggested that the difference between the anemia and the trait is qualitative and not simply one of degree.

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# USE OF THYMOL TURBIDITY AS LIPID ABSORPTION TEST

## EXPERIENCES WITH THYMOL TURBIDITY AND ZINC SULFATE TURBIDITY TESTS UNDER PHYSIOLOGIC AND PATHOLOGIC CONDITIONS

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THYMOL turbidity<sup>1</sup> results from an interplay of several factors. These are (a) reduction of serum albumin,<sup>2</sup> (b) increase of either the gamma<sup>1</sup><sup>3</sup> or beta<sup>4</sup> globulin fraction or more probably of lipid protein complexes electrophoretically migrating with the latter and/or (c) increase of lipids, especially phospholipids.<sup>5</sup> An incidental observation that the thymol turbidity increases significantly in normal dogs after meals suggested that the influence of ingested lipids or substances promoting lipid absorption upon thymol turbidity be investigated in normal subjects and in patients with various diseases. For comparison, the effect of lipid intake upon the blood total lipids, phospholipids and zinc sulfate turbidity was studied. The latter was recently described by Kunkel<sup>6</sup> as an index of serum gamma globulin concentration.

This study was planned to supply information as to whether the thymol turbidity could be adopted as a test for lipid absorption. It also provided additional observations as to the differential diagnostic value of thymol and zinc sulfate turbidity in liver disease.

### MATERIAL AND METHOD

Blood specimens were obtained from 471 patients. Some of them suffered from liver diseases as confirmed by a series of liver function tests and/or liver biopsies. Others had various other internal diseases. A third group was admitted to the hospital for conditions not internal in nature, for example fractures, simple hernia, small lacerations (control patients). On all specimens, the thymol turbidity was determined according to MacLagan.<sup>1</sup> It was expressed in units derived from a curve calibrated with barium sulfate.<sup>7</sup> In 114 cases, determination was made of the turbidity which developed after dilution of the serum with a buffered 2.4 mg. per cent zinc sulfate solution and in sixty cases after dilution with either the phenolbarbital buffer or distilled water alone. In five patients, determination was also made of the total serum lipids according to Boyd<sup>8</sup> and of the phospholipids as phosphates according to Fiske and Subbarow<sup>9</sup> after extraction according to Bloor.<sup>10</sup> The determination of the thymol turbidity was repeated in 165 cases, three and six hours following the ingestion of various lipids and/or 6 Gm. of choline hydrochloride in elixir form.\* In some patients the test was performed after the intake of different types of lipids. The turbidity produced by serum dilution with zinc sulfate, distilled water or a buffer solution was also measured in a smaller number of cases after lipid intake. The repeat determinations were made three and six hours after lipid intake because of previous experiences with vitamin A tolerance curves.<sup>11</sup>

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In confirmation of this, the maximal elevation of the thymol turbidity was found between three and six hours in five experiments in which blood was drawn hourly for six hours. The difference between the fasting level and the highest level after ingestion of lipids (maximal rise) was expressed as absolute difference or in per cent of the prelevel, respectively. For the analysis of the prevalue in the different clinical conditions, only the first determination on each patient was utilized. For determination of statistically significant differences the T value was determined according to the following formula:

$$t = \frac{\bar{x} - \bar{x}'}{s} \sqrt{\frac{(N_1 - 1)(N_2 - 1)}{N_1 + N_2 - 2}}$$

$\bar{x}$  = mean of one sample  
 $\bar{x}'$  = mean of second sample  
 $N_1$  = number in first sample  
 $N_2$  = number in second sample  
 $s$  = pooled standard deviation

A T value above 2.5 indicated a statistically significant difference.

In ten cases the thymol turbidity was determined before and after lipid intake on sera (1 ml) extracted once or several times with 5 ml of petrol ether or ethyl ether, similarly on sera (1 ml) to which 20, 40, or 100 mg of heparin were added, respectively, and on plasma from heparinized blood.

## RESULTS

*Thymol and Zinc Sulfate Turbidity in Various Pathologic Conditions*—The thymol turbidity varied in control patients from 0.5 to 6.9 units with an average of 2.1 (Table I). In only four instances was it above 4 units, which is usually considered as the upper limit of the normal. The average was slightly higher in patients with nephritis, heart disease, cachexia, infections and malignant tumors other than those of the biliary tract. In the latter three groups, the range extended occasionally into highly abnormal values. The average in gastrointestinal diseases was in the pathologic range, however, the statistical difference from the normal was not significant. Nonjaundiced patients with fatty livers (established by liver biopsy) had a normal turbidity. The average turbidity in acute hepatitis with jaundice was markedly elevated, the difference being statistically impressive. In only 7.9 per cent of the 126 cases of hepatitis was the turbidity below 4 units, however, some of these patients had almost completely recovered. The average in the nonicteric patients with cirrhosis was somewhat lower than in those with acute hepatitis though the difference from the norm was still statistically significant. In only 20.5 per cent of the nonicteric patients with cirrhosis was the turbidity below 4 units. The results in patients with cirrhosis with jaundice simulated those of acute hepatitis, only 12.9 per cent had a turbidity below 4 units. The cases of cirrhosis (with or without jaundice) which had a normal thymol turbidity were not necessarily less active than those with a high turbidity. In the biliary type of hepatitis produced by prolonged extrahepatic biliary obstruction, the average was only slightly elevated. Nevertheless, the difference from the normal was just statistically significant, a turbidity above 7 units was found in only 11.5 per cent. In the purulent type of hepatitis in which extrahepatic biliary obstruction was complicated by bacterial infection of the portal triads, the average turbidity was definitely above normal. The statistical difference from the normal was quite significant. In this group of seventeen cases, only in two was the turbidity normal and in five it was below 7 units.

TABLE I AVERAGE LEVEL, RANGE AND STATISTICAL SIGNIFICANCE OF ABERRATION FROM NORMAL OF THYMOL AND ZINC SULFATE TURBIDITY IN HOSPITAL CONTROLS AND PATIENTS WITH VARIOUS DISEASES

DIAGNOSIS	THYMOL TURBIDITY				ZINC SULFATE TURBIDITY			
	NUMBER OF CASES	AVERAGE	RANGE	T VALUE IN COMPARISON WITH HOSPITAL CONTROLS	NUMBER OF CASES	AVERAGE	RANGE	T VALUE IN COMPARISON WITH HOSPITAL CONTROLS
Control patients	64	2.1	0.5-6.1		3	7.1	1.9-18.5	
Nephritis	4	2.5	1.6-3.9	0.1	3	2.6	0.5-4.0	0.9
Heart diseases	8	2.9	0.5-7.0	0.4	2	10.7	3.1-16.1	0.4
Cachexia	17	2.9	0.9-7.1	0.5	8	11.3	5.3-22.6	1.9
Infections	16	3.1	1.1-8.7	0.7	2	11.6	9.9-19.2	1.0
Malignant tumors without involvement of the biliary tract	10	5	1.1-13	1.0	5	14.5	5.1-41.6	1.4
Gastrointestinal diseases	6	5.2	0.4-17.4	1.4	4	14.8	3.4-31.6	1.4
Fatty livers	6	3.1	1.0-5	0.6	0			
Acute hepatitis	126	11.7	1.7-29.2	2.1	15	17.8	6.8-52	3.4
Cirrhosis without jaundice	39	6.3	1.2-17.4	4.1	12	14.9	5.0-25.2	2.2
Cirrhosis with jaundice	91	10.1	1-68	9.7	15	21.2	2.4-59.2	4.5
Biliary hepatitis	69	4.4	0.6-14.6	2.8	13	16	2.7-17.0	0.2
Purulent hepatitis	17	13.0	3.5-25.5	7.8	2	4.5	4.0-5.0	0.2

The zinc sulfate turbidity revealed in normal subjects an average of 8 units, it was above 10 units in five out of thirty three cases. Slightly higher values were obtained in patients with heart disease. An average of about 15 units was obtained in infections, cachexia, gastrointestinal diseases, malignant tumors and emihosis without jaundice. In patients with acute hepatitis and especially in emihosis with jaundice the average elevation was even higher. This elevation was statistically significant. In 20 per cent of patients with acute hepatitis and in one third of those with emihosis and jaundice the zinc sulfate turbidity was below 10 units. In both forms of secondary hepatitis (biliary and purulent) the average turbidity was normal being above 10 units in only 13.3 per cent of such cases.

*Influence of Intake of Various Lipids Upon Thymol Turbidity*—After the intake of almost every type of lipid, the thymol turbidity rose moderately in control patients, but only slightly in patients with liver disease (Table II). The zinc sulfate turbidity rose also but far less than the thymol turbidity when expressed in percentage of the prevale. Thymol flocculation was not produced by administration of any of the lipids. The highest rise was encountered after intake of 50 Gm of butter. Some orientation experiments showed that the addition of 6 Gm of choline to 50 Gm of butter produced an even higher rise of the thymol turbidity than butter alone. Therefore the effect of butter, butter with choline, and choline alone was compared in another series of patients with various diseases (Table III). Butter and choline produced usually a greater elevation of the thymol turbidity than butter alone. However this

did not occur in all cases. Choline alone elevated the thymol turbidity significantly less than butter with choline or butter alone. The response of the thymol turbidity to butter and choline intake, alone or together, was far less marked in patients with liver disease.

TABLE II INFLUENCE OF ADMINISTRATION OF VARIOUS LIPIDS UPON THE THYMOL TURBIDITY

SUBSTANCE	AMOUNT	HOSPITAL CONTROL PATIENTS			PATIENTS WITH LIVER DISEASE		
		AVERAGE MAXIMAL RISE			AVERAGE MAXIMAL RISE		
		NUMBER OF CASES	IN UNITS	IN % OF PREVALUE	NUMBER OF CASES	IN UNITS	IN % OF PREVALUE
Butter	50 Gm	21	3.2	151.0	10	1.3	22.8
Egg yolks	4	10	1.6	106.8	13	2.8	37.2
Cholesterol	5 Gm	3	2.2	57.3	2	1.4	34.4
Lecithin	30 Gm	2	1.7	72.0			
Cod liver oil	30 c.c.	2	0.8	26.0			
Vitamin A in oil	100,000	2	0.2	5.1			
Vitamin A in aqueous dispersion	100,000	2	1.5	34.6			
Corn oil	50 c.c.	4	1.5	156.9			
Corn oil plus lecithin	50 c.c. plus 30 Gm	2	1.8	20.4			
Corn oil plus choline	50 c.c. plus 6 Gm	2	2.2	17.3			

TABLE III COMPARISON OF THE RISE OF THYMOL TURBIDITY FOLLOWING ADMINISTRATION OF 50 GM. OF BUTTER WITH AND WITHOUT 6 GM. CHOLINE AND OF 6 GM. CHOLINE ALONE IN PATIENTS SUFFERING FROM VARIOUS DISEASES

DIAGNOSIS	NUMBER OF CASES	BUTTER & CHOLINE		BUTTER			CHOLINE		
		MAXIMAL RISE IN UNITS		MAXIMAL RISE IN UNITS			MAXIMAL RISE IN UNITS		
		AVERAGE	RANGE	AVERAGE	RANGE	T VALUE IN COMPARISON WITH BUTTER AND CHOLINE	AVERAGE	RANGE	T VALUE IN COMPARISON WITH BUTTER AND CHOLINE
Normal subjects and control patients	20	4.2	1.3-9.8	2.9	0.7-6.5	2.1	1.1	0.3-3.1	4.9
Acute hepatitis	4	4.5	2.7-7.7	2.8	1.5-3.8	1.4	2.8	1.9-4.4	1.4
Cirrhosis	3	0.8	0.2-0.9	0.3	0.1-0.4	1.8	0.7	0.3-1.3	0.5
Obstructive jaundice	2	0.4	0.3-0.4	0.2	0.2-0.2		0.2	0.1-0.2	

*Influence of Intake of Butter and Choline Upon Blood Lipids and Turbidity Tests*—The thymol turbidity, phospholipids and total lipids rose in three control patients, the maximum elevation occurring three hours after the intake (Fig. 1). The zinc sulfate turbidity rose to a lesser degree. The slope of the rise was the steepest in the thymol turbidity, less in the serum phospholipids, and still less in the total lipids. All four factors rose insignificantly in the two patients with cirrhosis. In one control patient, the difference between the response to butter and choline, on one hand, and butter or choline alone, on the other hand, (previously described for the thymol turbidity) was mirrored by

the phospholipids, but not by the total lipids or the zinc sulfate turbidity (Table IV) In the two patients with cirrhosis (only one recorded in Table IV) the rise in all instances was insignificant and did not permit comparison

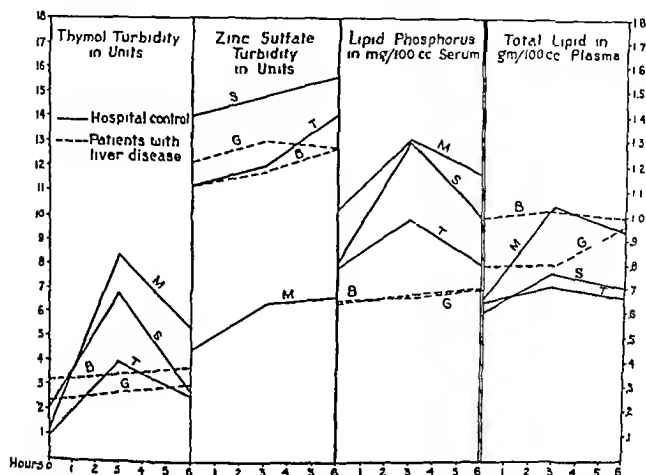


Fig 1—Response of thymol turbidity zinc sulfate turbidity serum lipid phosphorus and plasma total lipids to the intake of 50 Gm of butter and 6 Gm of choline

TABLE IV COMPARISON OF THE RISE OF THYMOL TURBIDITY, ZINC SULFATE TURBIDITY, SERUM LIPID PHOSPHORUS AND TOTAL LIPIDS AFTER ADMINISTRATION OF 50 GM BUTTER TOGETHER WITH 6 GM CHOLINE AND AFTER BUTTER OF CHOLINE ALONE IN A HOSPITAL CONTROL AND IN A PATIENT WITH CIRRHOSIS

TEST	CONTROL PATIENT				CIRRHOSIS			
	PRE VALUE	MAXIMAL RISE AFTER ADMINISTRATION OF			PRE VALUE	MAXIMAL RISE AFTER ADMINISTRATION OF		
		BUTTER+ CHOLINE	BUTTER	CHOLINE		BUTTER+ CHOLINE	BUTTER	CHOLINE
Thymol turbidity in units	23	48	35	10	32	07	05	02
Zinc sulfate turbidity in units	47	20	22	17	112	12	10	06
Lipid phosphorus in mg/100 c.c. serum	02	51	30	25	05	06	07	07
Total lipids in gm/100 c.c. plasma	077	018	025	011	098	004	005	005

*Response of Thymol and Zinc Sulfate Turbidities to the Simultaneous Intake of 50 Gm of Butter and 6 Gm of Choline in Various Pathologic Conditions*—The rise of the thymol turbidity in control patients was on the average 31 units or 237.4 per cent. The rise was below 1 unit in only three out of thirty-nine instances (Table V). The same response was found in neurologic conditions and in a few cases of nephritis. The rise in thymol turbidity was

TABLE V MAXIMAL RISE OF THE THYMOL AND ZINC SULFATE TURBIDITY IN HOSPITAL CONTROLS AND PATIENTS WITH VARIOUS DISEASES FOLLOWING THE INTAKE OF 50 GM BUTTER AND 6 GM CHOLINE

DIAGNOSIS	MAXIMAL RISE IN THYMOL TURBIDITY					MAXIMAL RISE IN ZINC SULFATE TURBIDITY				
	NUM BER OF CASES	IN UNITS		T VALUE FROM HOSPITAL CONTROLS	AVER AGE IN %	NUM BER OF CASES	IN UNITS		AVER AGE IN %	
		AVERAGE	RANGE				AVERAGE	RANGE		
Control patients	59	3.1	0.4-8.8		237.4	12	2.8	0.7-6.8	34.5	
Neurologic diseases	11	3.0	1.2-6.4	0.8	107.4	10	1.9	0.7-5.6	57.6	
Cardiac diseases	8	0.9	0.3-2.2	4.5	68.1	2	0.7	0.5-2.2	5.3	
Infections	16	1.3	0.4-3.4	4.9	58.0	2	1.1	0.7-1.4	8.9	
Carcinoma without involvement of biliary tract	10	1.3	0.1-3.2	4.2	41.0	5	1.3	0.7-1.6	26.6	
Wasting diseases	13	1.6	0.2-2.8	4.0	59.8	8	2.3	0.4-6.8	32.1	
Gastrointestinal diseases	6	0.6	0.3-2.0	4.5	19.5	4	0.7	0.5-0.9	16.9	
Nephritis	4	2.3	0.1-4.1	1.5	105.6	3	0.8	0.3-0.9	95.2	
Acute hepatitis	16	2.5	0.2-7.7	2.1	18.4	4	1.7	0.8-3.6	11.2	
Cirrhosis without jaundice	16	1.3	0.2-3.6	5.0	27.9	8	1.1	0.3-2.9	13.4	
Cirrhosis with jaundice	18	1.0	0.1-3.2	5.9	18.0	10	0.8	0.4-2.1	9.8	
Obstructive jaundice	8	0.8	0.1-2.4	4.9	25.3	4	0.5	0.2-2.8	10.9	

significantly less in patients with infections, carcinomas (other than those involving the biliary tract) and cardiac or wasting diseases, the average being around 1 unit. The smallest response was noted in gastrointestinal diseases in which the turbidity rise was only about 0.6 unit. The response was also depressed to a statistically significant degree in liver diseases with the exception of acute hepatitis. If expressed in per cent, the lack of rise was especially marked in liver diseases in view of their elevated prevalues. In patients with acute hepatitis, who usually had a high prevalue, the absolute rise was nearly normal, but the percentage rise was almost the lowest recorded. The rise was slight, whether comparisons were made in units or percentages, in cases of cirrhosis with jaundice and in obstructive jaundice. This was so despite frequent elevation of the prevalues. In general, a rise below 1.5 units suggested an aberration from the normal. Elevations of less than 1.0 unit pointed to a pathologic condition. As a general trend, low fasting levels of the thymol turbidity were associated with a significant response to administration of butter and choline. Patients with high fasting levels showed, as a rule, little response (Fig. 2). An exception to this was noted in acute hepatitis in which an almost normal response occurred in face of a high fasting turbidity. Another exception was seen in gastrointestinal disturbances in which both fasting level and response were low.

The zinc sulfate turbidity responded similarly to the thymol turbidity, but its rise was less marked whether in units or in per cent of the preliminary value. The low turbidity readings of serum dilutions with either distilled water or sodium barbitol-barbitol buffer without thymol were only slightly (not over 0.2 unit) if at all influenced by lipid intake.

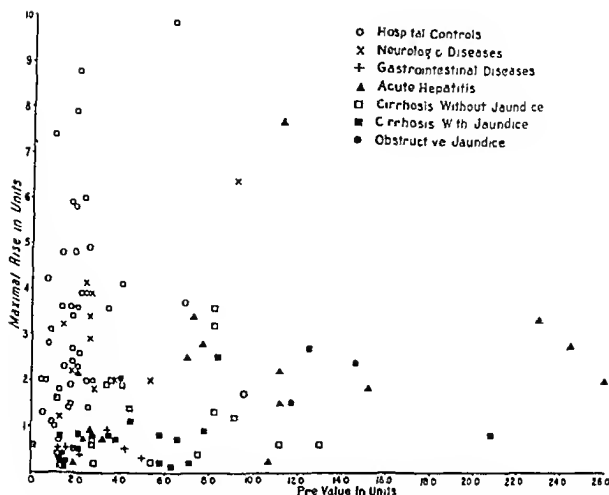


Fig 2—Plot of the thymol turbidity before lipid administration with the maximal rise following administration of 50 Gm butter and 6 Gm choline in hospital controls and patients suffering from various diseases

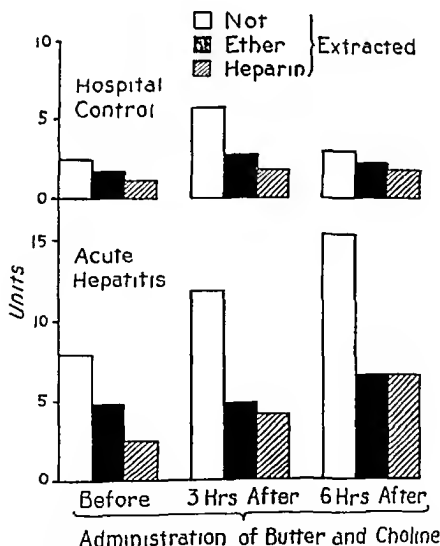


Fig 3—Influence of adding of heparin to the serum or extracting it with ethyl ether upon the thymol turbidity in a hospital control and a patient with acute hepatitis before and after administration of 50 Gm butter and 6 Gm choline



*Influence of Lipid Solvents Upon Thymol Turbidity*—As observed in at least twenty-five cases of each group, the thymol turbidity, normal or elevated due to either disease or intake of butter and choline, was not altered by extraction of the serum with petrol ether, but was markedly reduced by extraction with ethyl ether (Fig 3). The thymol turbidity was reduced in serum to which heparin was added as well as in plasma from heparinized blood independently of pathologic or postprandial elevation. This reduction was augmented by increasing the amount of heparin used or repeating the ethyl ether extraction.

#### DISCUSSION

The diagnostic value of the thymol turbidity test, reported by many investigators,<sup>1, 12, 20</sup> has been confirmed in this study. The turbidity was occasionally elevated in various conditions with possible secondary involvement of the liver, for example general infections, malignant tumors and gastrointestinal diseases, however, it was regularly and markedly elevated in acute hepatitis. It remained normal in patients with fatty livers. In cirrhosis with jaundice the turbidity was usually slightly lower, and in cirrhosis without jaundice, it was markedly lower than in acute hepatitis. In contrast, extrahepatic biliary obstruction, even of prolonged duration, usually revealed a normal turbidity. This difference was especially noticeable if the border line was drawn at 7 units. However, if a bacterial infection involved the portal triads, for example cholangitis, complicated extrahepatic biliary obstruction (purulent hepatitis<sup>17</sup>), the thymol turbidity became markedly elevated, as a rule, to over 7 units. The difference between elevated thymol turbidity in primary hepatitis or cirrhosis and normal turbidity in extrahepatic biliary obstruction even with liver damage is of practical importance.<sup>21</sup> This difference holds true only in the absence of bacterial infection of the portal triads.<sup>16</sup>

The demonstrated difference in the results of the thymol turbidity test in various conditions is apparent in the mean or in a statistical evaluation of a larger number of cases but may not necessarily hold true for individual patients. Thus, a normal thymol turbidity may occasionally be found in hepatitis or cirrhosis with marked jaundice when other hepatic tests are abnormal. Conversely, the thymol turbidity may be elevated in extrahepatic jaundice and even in diseases not primarily hepatic such as rheumatoid arthritis.<sup>24, 25</sup>

Elevation of the zinc sulfate turbidity was found not only in hepatic diseases but to almost the same degree in other inflammatory and neoplastic conditions with little or no liver damage. In contrast, it was normal in the cases of extrahepatic biliary obstruction. This latter observation, if confirmed on a large number of cases, would make this test of great value in the differentiation between extrahepatic biliary obstruction and primary hepatitis and cirrhosis. The significance of the zinc sulfate turbidity test thus appears to lie in its failure to rise in extrahepatic jaundice. The zinc sulfate turbidity was especially high in cirrhosis. As a rule, markedly elevated zinc sulfate turbidity in face of only moderately elevated thymol turbidity suggests cirrhosis. On the other hand, high thymol turbidity with moderately elevated zinc sulfate turbidity speaks for an acute hepatitis.

The transient rise of the thymol turbidity after lipid intake (especially after butter and eboline) may have diagnostic significance because it parallels the rise of the blood total lipids and especially of the phospholipids. That the postprandial rise of the thymol turbidity is due to serum phospholipid elevation is also indicated by the high phospholipid concentration of the precipitate produced by adding thymol to serum. This hypothesis is also supported by the observation that choline, which is essential in lecithin formation, may enhance the effect of butter ingestion upon the thymol turbidity, and the serum phospholipid level. Furthermore, the administration of choline alone increases the thymol turbidity.

The rise of the thymol turbidity after butter alone may result from a combination of fats with phospholipids during absorption,<sup>1,10</sup> although such a combination is still controversial.<sup>2</sup> Increase of the butter effect by choline could be ascribed to a role of choline in the fat transport through the intestinal wall,<sup>6,27</sup> similar to the function of choline in fat mobilization from the liver.<sup>28,29</sup>

The elevation of the thymol turbidity induced by fat ingestion and the spontaneous elevation in pathologic conditions like acute hepatitis are equally lowered by ethyl ether but not by petrol ether extraction of the serum.<sup>6</sup> Both elevations are equally depressed *in vitro* by heparin which is known to abolish visible lipemia, at least *in vivo*.<sup>30,31</sup> That does not necessarily imply that the increase in the thymol turbidity in liver diseases is primarily due to a lipid factor. Thus, Kunkel and Hoagland<sup>2</sup> have shown that at least in some stages of acute hepatitis the thymol turbidity elevation depends primarily on protein changes.

The response of the thymol turbidity to the administration of butter and choline can be considered as an index of intestinal lipid and most probably phospholipid absorption. The rise is only slight in many conditions in which a disturbance of fat absorption can be expected as in gastrointestinal or in severe wasting diseases. Poor response may be found in obstructive jaundice or in hepatic diseases, for example cirrhosis, which have a high spontaneous thymol turbidity. Such a minimal rise is not necessarily caused by liver damage as such, in view of the almost normal response in infectious hepatitis. It is more likely due to associated gastrointestinal disturbances. The response of the thymol turbidity to lipid intake represents thus a technically simple test for intestinal lipid absorption which may be of diagnostic value.

#### SUMMARY

In confirmation of previous reports, the thymol turbidity test was found elevated in acute hepatitis, less so in cirrhosis with and without jaundice. It was normal in patients with fatty livers without jaundice, slightly elevated in obstructive jaundice without infection of the biliary tract but elevated when bacterial infection was present. Occasionally, elevation was found in diseases without apparent liver involvement while in rare instances of even severe primary liver cell damage the turbidity was normal.

The zinc sulfate turbidity was found elevated in liver diseases and in inflammatory or malignant conditions in general but was normal in cases of extrahepatic obstructive jaundice. Therefore, a low zinc sulfate turbidity is of great diagnostic importance. The ratio between thymol and zinc sulfate turbidity is of practical significance in the differentiation of acute hepatitis and cirrhosis.

Administration of various lipids raised the thymol turbidity. For this purpose, 50 Gm butter given together with 6 Gm choline are especially efficient. The rise of the thymol turbidity was parallel with that of the serum phospholipids, but less so with that of the total plasma lipids and the zinc sulfate turbidity. Butter or choline alone usually produced a less marked rise than butter and choline together.

The spontaneous elevation of the thymol turbidity produced by pathologic conditions as well as the rise caused by the intake of lipids is equally reduced *in vitro* by adding heparin to the serum or by extracting serum with ether but not with petrol ether. The rise in the thymol turbidity after the ingestion of fat was markedly depressed in gastrointestinal diseases, obstructive jaundice and cirrhosis. The depression in wasting diseases was of lesser degree. In acute infectious hepatitis the postprandial rise was almost normal. The response of the thymol turbidity to the intake of butter and choline may serve as a simple clinical test of intestinal absorption of fat.

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# THE BOUND GLUCOSAMINE OF SERUM MUCOID IN DIABETES MELLITUS, FLUCTUATIONS OBSERVED UNDER THE INFLUENCE OF INSULIN

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MANY authors have speculated upon the metabolic disorder associated with the carbohydrate defect in diabetes mellitus. This paper presents data obtained in a study of the concentration of glucosamine in the blood of diabetic and other patients.

Glucosamine is thought at present to occur in blood in the form of a polysaccharide composed of acetylglucosamine, mannose and galactose, bound to a protein molecule of the plasma, the whole complex is termed a mucoid, or a seromucoid.<sup>1</sup> Ordinary methods of precipitating plasma proteins, as by salting out or by treating with alcohol, coprecipitate the seromucoids with the albumin and globulin. A useful way of separating them is to acidify the plasma slightly and then to heat it briefly<sup>1</sup> the albumins and globulins coagulate promptly, leaving the serum mucoids in the clear liquid.

## METHOD

A few simple preliminary studies sufficed to devise a process for routine preparation of blood samples for this preliminary survey. The following procedure was used. One milliliter of ovalated plasma was mixed with 3 ml of 0.01N HCl in a 15 ml conical centrifuge tube. The tube was placed in a boiling water bath for three to four minutes while its contents were kept in motion. Coagulation occurred rapidly. The tube was then cooled and centrifuged. The supernate was removed and placed in a 5 ml glass ampul, and the ampul sealed with a blowlamp. The sealed ampul was heated in a boiling water bath for twenty four hours to liberate glucosamine from the polysaccharide complex.

Color was produced according to the method of Elson and Morgan,<sup>2</sup> following the additional directions of Palmer, Smyth, and Meyer.<sup>3</sup> The following reagents are required (all should be kept in icebox)

1 Two per cent acetylacetone in 0.5N Na<sub>2</sub>CO<sub>3</sub>. Add 0.2 ml acetylacetone to 10 ml 0.5N Na<sub>2</sub>CO<sub>3</sub>, and dissolve by shaking. Prepare fresh every day or two.

2 *p*-Dimethylaminobenzaldehyde reagent. The substance must be purified before use.<sup>2, 3</sup> Dissolve 0.8 Gm in 30 ml aldehyde free alcohol and add 30 ml concentrated HCl.

3 Aldehyde free alcohol. This is prepared by distillation of absolute alcohol after treatment with Ag<sub>2</sub>O and sodium hydroxide,<sup>3</sup> and diluting to make 95 per cent.

4 Glucosamine standard. Twice recrystallized glucosamine HCl is dissolved in water saturated with chloroform to make 20 mg per 100 milliliters.

The sealed ampul was opened after twenty four hours of heating and the contents were filtered. One milliliter of the filtrate was placed in a test tube marked at 10 milliliters. One milliliter of the acetylacetone reagent was then added, and the sides of the tube were washed down with 1 ml of water. A standard was set up in the same manner, using 1 ml of the standard solution. A blank was made using 1 ml of water, but otherwise treated exactly as the test and standard mixtures. The tubes were heated in a boiling water bath for fifteen minutes, then cooled. Approximately 2 ml of aldehyde-free alcohol were added to each tube, following

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which 3 ml of p dimethylaminobenzaldehyde reagent were added to each. Then aldehyde free alcohol was added to each tube to the 10 ml mark, the contents of each were mixed thoroughly and all were set aside for forty five minutes for the colors to develop and for bubbling to cease.

Color comparisons were made in a Coleman spectrophotometer. A wave length setting of .35 was found to be most sensitive to the color, and was therefore used throughout. The blank solution was first employed to establish the blank value, after which the standard and the test solutions were measured in the machine. The glucosamine content of the test sample was read from a calibration curve previously established for the instrument and for the method through a range of known glucosamine concentrations from 5 to 100 mg per cent. The standard solution served to verify this curve each time in the neighborhood of 20 mg per cent. Whenever the test sample showed unusually high values, it was customary to repeat the determination after diluting the original filtrate enough to bring the concentration well below 40 mg per cent.

In passing it should be mentioned that the method employed was designed to measure the glucosamine that is easily separable hence the values found represent only this portion. More drastic methods of hydrolysis and use of whole plasma will doubtless give higher values for "total" glucosamine.

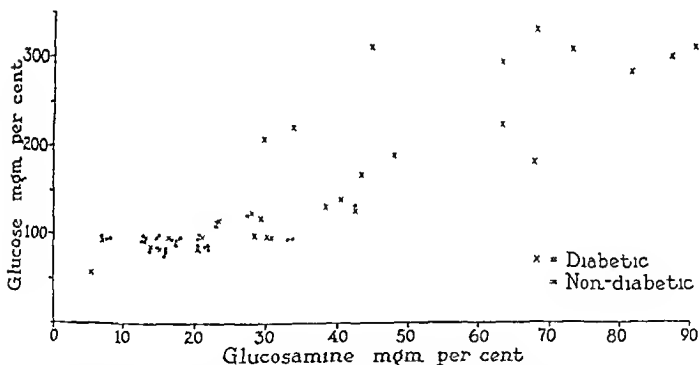


Fig 1—A scatter-diagram to show the correlation between blood glucose and blood glucosamine in nondiabetic and in diabetic subjects. This diagram presents the data given in the tables.

## RESULTS

Three hundred and seventy eight determinations were made upon 250 subjects. It was soon obvious that plasma glucosamine followed the blood sugar level and that the findings of greatest interest would be made upon diabetic blood. In the tables the data are separated into two categories: diabetic and nondiabetic. The examples offered were selected because blood sugar determinations were made with the glucosamine studies; those left out had no blood sugar determinations, but the glucosamine levels were not unique in any way. The values offered in the tables appear in the scatter diagram (Fig 1).

TABLE I PLASMA GLUCOSAMINE CONTENT AND BLOOD SUGAR LEVELS IN NONDIABETIC SUBJECTS

SEX AND AGE		GLUCOSAMINE (MG %)	GLUCOSE (MG %)	CLINICAL STATE
<i>0.9 mg per cent</i>				
M	46	2.7	83	Cholelithiasis, icteric index 7.0
M	62	4.2	105	Hypertension
M	33	5.5	83	Functional bowel distress
F	59	6.8	99	Incisional hernia
F	61	7.4	91	Chronic hypertrophic arthritis
M	47	8.0	96	Heart block
F	61	8.1	80	Acute appendicitis, WBC 15,000
F	18	8.3	86	Cellulitis of left axilla
<i>10.19 mg per cent</i>				
F	84	10.7	99	Glomerulonephritis
M	40	11.7	86	Ulcerative colitis
M	18	12.8	94	Generalized peritonitis, WBC 12,400
F	69	12.8	99	Myocardial infarction
M	49	13.3	99	Cholelithiasis, icteric index 7.2
F	33	13.9	83	Pregnancy, fibroid tumor
F	40	14.8	98	Hypothyroidism
M	74	15.0	85	Carcinoma of pancreas
F	75	15.0	100	Hypertension, senility
F	49	15.8	77	Urethral polyps
F	63	16.0	84	Hypothyroidism
M	39	16.0	106	Catarrhal jaundice, icteric index 13.1
M	29	16.0	81	Pilonidal abscess
M	41	16.0	83	Glomerulonephritis
M	85	16.7	95	Generalized arteriosclerosis
M	64	17.5	89	Ménière's syndrome
F	24	18.1	81	Herpetic iritis
M	40	18.1	99	Suspected mebiasis
M	38	19.0	92	Chronic mastoiditis
M	80	19.3	79	Prostatic hypertrophy
<i>20.26 mg per cent</i>				
F	59	20.5	87	Atypical pneumonia
F	38	20.5	100	Acute back strain
F	68	20.5	94	Acute bronchitis, hypertrophic arthritis
M	65	21.1	93	Emphysema
F	55	21.5	86	Menopausal bleeding
M	74	21.8	84	Prostatic hypertrophy
M	58	21.8	97	Psychoneurosis
M	86	21.8	87	Arteriosclerotic heart disease
F	43	23.0	116	Multiple sclerosis
M	62	25.3	98	Hypertensive cardiovascular disease
F	48	26.0	99	Chronic cervicitis
M	53	26.0	116	Hypertensive cardiovascular disease
<i>27 mg per cent and over</i>				
F	67	27.4	123	Arteriosclerosis, cholecystitis
M	67	31.2	106	Carcinoma of prostate with metastases
F	30	31.7	156	Tubal pregnancy, sulfadiazine 3.4 mg %
M	72	33.1	95	Adenocarcinoma of prostate
M	78	33.1	133	Prostatic hypertrophy
F	80	34.7	96	Hypertensive cardiovascular disease
M	72	34.7	121	Skull fracture
M	53	38.9	116	Hypertensive cardiovascular disease
M	69	42.4	135	Prostatic hypertrophy
M	77	43.2	101	Fracture, arteriosclerosis
F	77	43.2	114	Pelvic abscess
M	55	52.8	105	Prostatic hypertrophy

TABLE II PLASMA GLUCOSAMINE CONTENT AND BLOOD SUGAR LEVEL AMONG DIABETIC PATIENTS

SEX AND AGE		GLUCOSAMINE		DATE
		(MG %)	(MG %)	
F	52	63.3	2.6	3/1
		132	167	3/8
		129	168	3/9
		40.3	141	3/11
M	68	110	133	2/4
		103	119	2/5
		19.3	132	2/16
		37.5	132	3/3
		74	132	3/5
F	61	11.7	53	2/27
		48.0	104	3/8
M	77	74	100	2/25
		139	87	2/27
F	55	48.0	160	3/9
F	70	11.9	110	2/24
		21.8	68	3/4
		3.0	106	3/9
F	43	03.3	296	2/24
		67.0	54	2/25
		30.5	98	2/26
		48.8	183	3/2
		24.1	98	3/4
F	23	12.8	120	2/9
		73.1	308	2/25
F	67	80.9	303	3/4
F	81	30.5	136	2/24
		48.8	175	2/27
M	52	29.2	154	3/10
F	59	32.5	115	2/3
		26.0	124	2/4
		39.5	132	2/5
		24.1	143	2/6
		28.6	138	2/7
F	67	29.2	167	2/5
		33.0	221	2/6
		29.8	209	2/11
		24.1	112	2/12
		16.5	94	2/16
		15.7	118	2/19
		21.1	97	2/24
		42.5	129	2/26
		7.4	48	2/12
		43.2	270	2/18
M	70	41.7	279	2/9
		27.4	107	2/11
		44.8	248	2/16
		41.7	220	2/17
		15.0	84	2/18
		12.8	94	2/19
		23.0	116	2/24
		28.0	125	2/26
		24.1	135	2/27

(Continued on following page)



TABLE II—CONT'D

SEX AND AGE		GLUCOSAMINE (MG %)	GLUCOSE (MG %)	DATE
F	72	39.6	196	2/26
F	59	35.3	133	3/11
F	27	18.1	56	3/4
		44.0	160	3/8
		36.7	133	3/9
		18.1	75	3/10
M	55	5.5	58	2/27
		29.2	119	3/1
		30.2	98	3/3
F	58	54.7	209	3/2
		48.0	191	3/4
		47.5	168	3/10
M	20	44.8	317	1/20
M	57	68.0	286	2/24
		20.5	87	2/27
F	82	54.7	270	1/21
M	57	32.5	132	3/11
		7.9	94	3/15
F	53	25.3	190	2/24
		68.0	331	2/25
		17.5	91	2/27
		58.5	185	3/9
		90.5	312	3/15
F	17	81.6	284	8 30 A M
		67.0	179	10 30 A M
		36.0	114	12 30 P M
		35.2	124	2 30 P M
		56.2	288	4 30 P M

The nondiabetic group was composed of 151 individuals, and their glucosamine levels ranged through the following concentrations

GLUCOSAMINE LEVEL	NUMBER OF INDIVIDUALS
17 to 10 mg per cent	29
11.0 to 20.0	73
21.0 to 26.0	37
27.0 and above	12

Although it is not possible to establish a "normal" range of glucosamine levels from this study because no normal subjects were included it seems likely that normal values by this method will lie in the neighborhood of 15 to 25 mg per cent. The lowest value found was 1.7 mg per cent, the highest among the nondiabetics, 52.8 mg per cent. Perhaps the only observation to be considered at this time is that the level seems to rise with age. Several patients under treatment with sulfadiazine gave levels above the normal, but the disease being treated may have been partly responsible for the effect. The high incidence of neoplasia and of cardiovascular disease in the group having the highest glucosamine levels among the nondiabetic subjects perhaps depends as much upon the advanced age of the subjects as upon their diseases.

The diabetic group (Table II)\* was composed of forty three individuals of which twenty six appear in the table. All were in the Hospital under treatment. Those left out resembled those shown in all essential respects. Two features are noteworthy: glucosamine levels far higher than occur among non-diabetic subjects are frequently encountered, and glucosamine levels parallel blood sugar levels with remarkable consistency. Glucosamine levels above 40 mg per cent are unusual among nondiabetic subjects, yet they are common in diabetic subjects. Levels as high as 90 to 95 mg per cent have been observed.

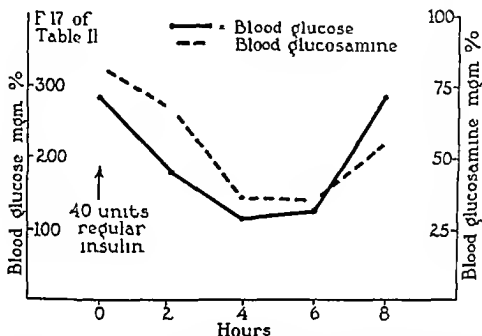


Fig. 17 — The response of blood glucosamine to insulin in a diabetic subject. The graph shows how both blood glucose and blood glucosamine fall after a dose of insulin in a young diabetic subject (F 17 of Table II). Note also that glucosamine rises with glucose as the insulin effect disappears.

in uncontrolled diabetes. The remarkable parallelism between glucosamine and glucose levels which is evident in Table II is not unvarying, divergences occur frequently enough to indicate that two phenomena are involved. Dissociation is often observed during insulin effect: the fall of glucosamine frequently lags behind the fall of blood sugar.

Glucosamine is obviously responsive to insulin. A single experiment was performed (F 17 in Table II) in which blood sugar and glucosamine were measured every two hours after an adequate dose of insulin had been given. Within four hours the blood sugar fell from 284 to 114 mg per cent, and during that interval glucosamine dropped from 81.6 to 36.0 mg per cent. Four hours later the blood sugar had risen again to 288 mg per cent, and during that time the glucosamine rose to 56.2 mg per cent. The correspondence is shown in the graphs in Fig. 2.

The association of glucosamine level with age of subject mentioned earlier in discussing nondiabetic data is not apparent among diabetic subjects, very high levels are encountered at all ages and so are low levels after insulin. The absence or presence of control of the diabetic state overshadows all other possible effects on the plasma glucosamine level in diabetic subjects.

## DISCUSSION

The experiences described in this paper indicate that the mucopolysaccharide of the mucoid fraction of blood plasma which yields glucosamine after mild acidic hydrolysis is somehow involved in the diabetic defect, and that it responds to insulin in roughly the same manner as glucose itself as far as the direction, speed, and magnitude of the response is concerned. The only earlier report to be found, that of West and co-workers,<sup>4</sup> states that glucosamine in diabetic blood is not elevated. These workers used another and more drastic method of hydrolysis, and they employed whole serum directly.

Nothing is known about why the mucopolysaccharide under discussion should appear in greater amounts in uncontrolled diabetes. Many substances appear in greater amounts under that circumstance, and many of them are responsive to the effect of insulin. A recent general theory of the function of insulin<sup>5</sup> postulates that the glucose anomaly in diabetes is but one manifestation of insulin deficiency, perhaps the phenomenon described in this paper is another.

## SUMMARY

1 A procedure is given by which easily separable serum mucoid glucosamine was studied.

2 Representative data for nondiabetic and for diabetic subjects are offered.

3 Insulin seems to exert a pronounced effect on the level of glucosamine found by the method described.

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# DIBUTOLINE AS AN ANTIDOTE FOR DIISOPROPYL FLUOROPHOSPHATE POISONING IN MICE

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THE toxicity of diisopropyl fluorophosphate (DFP), an anticholinesterase agent, has been described by a number of authors. The early reports which established the mode of action and toxicity of DFP are not generally available but are quoted in several published articles. Scholz<sup>1</sup> reported that topical application in the eye could produce death in rabbits which resembled eserine poisoning. McNamara, Koelle, and Gilman, and also, Modell and Klop<sup>2</sup> pointed out that DFP, an anticholinesterase is toxic by a combination of nicotinic and muscarinic actions. No single drug is known which will protect from both of these actions. Atropine provides various degrees of protection against the muscarinic effects of DFP or the closely related dimethyl fluorophosphate in cats, rabbits, and mice.<sup>1, 2, 3, 4</sup> In cats and mice this antidote is moderately effective since the muscarinic action is the dominant one. It is much less effective in the rabbit where the nicotinic effect predominates. These same authors reported that magnesium sulfate counteracts the nicotinic effects of DFP. Salts of calcium, aluminum and gold, according to Modell, also have some "anti nicotinic action." Magnesium sulfate alone seldom prevented death in the animal administered DFP, but when it was combined with atropine the combination gave greater protection than either drug alone. Whereas it was to be expected that the combination of two anticholinesterase agents would increase the toxicity, Koster<sup>5</sup> found that this was not true when physostigmine was administered before DFP. Physostigmine protected from a subsequent lethal dose of DFP. DFP given before physostigmine increased the toxicity of physostigmine. This seemingly paradoxical result was further studied and explained by Koelle<sup>6</sup> who classified nineteen other anticholinesterase agents for their protective action against DFP.

Acetyl beta methylcholine protects mice from inhaled dimethyl fluorophosphate but not from parenteral injections. This protection apparently is due to local action on the respiratory tract which alters the rate of absorption of the inhaled drug.<sup>4</sup>

Seeking a less diastolic cycloplegic agent than atropine for ophthalmologic use, Swan and White<sup>7</sup> synthesized a series of choline esters which have blocking rather than stimulating effects on the parasympathetic system. Dibutoline<sup>8</sup> (dibutyl urethane of dimethyl ethyl  $\beta$  hydroxyethyl ammonium sulfate) was found to be the most suitable compound of the group. The general pharmacology of dibutoline has been studied by Featherstone and White<sup>9</sup> and by Peterson and Peterson<sup>10</sup> who have definitely established the blocking action on the parasympathetic system. Pfeiffer<sup>11</sup> has suggested that the atropine like

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action of dibutoline may be due to a similar umbrella-like spacial arrangement of prosthetic groups in the molecule

The similarity of action of dibutoline to atropine raised the question as to whether dibutoline would have a protective effect against DFP. The present study is an attempt to evaluate dibutoline as an antidote for DFP poisoning.

#### EXPERIMENTAL METHODS

Three groups of mice, averaging 25 grams each, were obtained and used for the study.\* Mice from each of these groups were given intraperitoneal injections of fresh DFP solution in Ringer's solution so prepared that the desired dosage of drug would be contained in 0.5 cc of solution. After the responses of a number of unprotected mice were observed, various dosages of atropine or dibutoline were given to other mice. One minute later these mice were injected with the same dosage of DFP used previously.

Control groups of mice were injected with various doses of dibutoline or atropine alone. When death occurred it was usually within thirty minutes, but any death within one week was assumed to be due to the medication.

#### RESULTS

The sensitivity to DFP varied in the different groups of mice, but each group in itself was quite consistent. In animals of the first group DFP 0.125 mg per animal produced a mortality of 38 per cent. See Table I. When atropine 0.02 mg per animal was administered one minute before the DFP the mortality was reduced to 12 per cent. This same dosage of atropine alone did not appear to affect the mice in any untoward way.

When dibutoline 2.5 mg per animal was injected alone it was found to cause 100 per cent mortality within ten minutes. As the dosage of dibutoline was decreased, the percentage mortality was decreased until with 0.313 mg per animal no deaths occurred. While dibutoline 1.25 mg per animal alone

TABLE I THE COMPARATIVE RATES OF PROTECTION PROVIDED FOR DFP BY DIBUTOLINE

	NUMBER OF MICE	DRUG (MG PER 25 GM ANIMAL)	NUMBER OF DEATHS	PERCENTAGE MORTALITY
Group A	39	DFP 0.125	15	38
	50	Atropine 0.02		
		DFP 0.125	6	12
	13	Dibutoline 2.50		
		DFP 0.125	13	100
	12	Dibutoline 2.50	12	100
	8	Dibutoline 1.25	7	87
	35	Dibutoline 0.625	18	51
	15	Dibutoline 0.313	0	0
	25	Dibutoline 1.25		
		DFP 0.125	10	40
	25	Atropine 0.02	0	0
Group B	24	Dibutoline 0.313		
		DFP 0.125	2	8
	90	DFP 0.125	89	99
	25	Dibutoline 0.313		
Group C		DFP 0.125	15	60
	23	DFP 0.250	14	61
	28	Dibutoline 0.313	0	0
	44	Dibutoline 0.313		
		DFP 0.250	13	30

\*Cats were first tried and then discarded as experimental animals because of the great variation in response to a given dosage and the large amounts of drug needed for each animal.

caused a mortality of 87 per cent when it was followed by DFP 125 mg per animal, this mortality was reduced to 40 per cent showing that DFP has some protective action against the lethal dosages of dibutoline. DFP did not protect from the  $LD_{100}$  dosage of dibutoline (250 mg per animal). When the sublethal dose of 0.313 mg per animal of dibutoline was administered before the DFP the mortality was reduced to 8 per cent.

The second group of mice obtained from a different source, were more sensitive to DFP. The dosage 0.125 mg per animal DFP caused a mortality of 99 per cent in a large group of mice. This was reduced to 60 per cent by dibutoline 0.313 mg per animal.

The third group of mice was less sensitive to DFP so that 0.250 mg per animal caused a mortality of 61 per cent. This was reduced to 30 per cent by the dibutoline 0.313 mg per animal.

Death from either drug usually occurred within the first fifteen minutes and was rare after the first forty eight hours. Thus dibutoline has a moderate degree of protective action against the toxic effects of DFP.

Analysis of these results to determine the probability that they might result from chance by the use of Pearl's formula for determining Chi square gave the following results. The probability of chance in group A is 1 to 110, group B, 1 to 500,000,000, and group C 1 to 80. These results, therefore, all fall within the definitely significant range.

#### SUMMARY

Dibutoline, a choline derivative with parasympathetic blocking and no stimulating action, was tested to determine if it would protect against diisopropyl fluorophosphate poisoning. Dibutoline was effective in protecting mice against DFP toxicity, as was atropine. Both drugs were moderately efficient as antidotes when their injection preceded that of DFP. DFP has some protective action against lethal doses of dibutoline.

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## PHARMACOLOGY OF ALLYLTHIOMETHYL- AND n-BUTYLTHIOMETHYLPENICILLIN

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CONTRARY to the original conception, penicillin is now recognized as a group of substances having a chemical and biologic similarity. It is therefore a generic name. Benzylpenicillin (penicillin G) is the best known and most widely used of the group. New biosynthetic penicillins<sup>1</sup> recently have become available through the addition of certain organic precursors to the culture medium. Although different penicillins show a similar specificity of action on various microorganisms, several of the new penicillins exhibit pharmacologic properties which appear to offer some advantages over those of penicillin G. In this communication, certain physiologic actions of two of these penicillins,<sup>2</sup> allylthiomethylpenicillin (AT) and n-butylthiomethylpenicillin (BT), are compared with those of penicillin G.

Particular interest in the toxicity of these new penicillins by intradural injection was aroused by a report of Baker<sup>3</sup> of our laboratories. In screening some thirty penicillins for their action against rabie virus in the mouse brain, Baker found it necessary to establish their relative intracerebral toxicity values. His results indicated that penicillins AT and BT were much less toxic than penicillin G.

Direct application of penicillin G to the cerebral hemispheres frequently incites convulsions,<sup>4,5</sup> and in large doses may lead to necrosis of the parts so exposed.<sup>6,7</sup> The effectiveness of the barbiturates in alleviating convulsions is well known. Sodium Amytal (Sodium Iso-amyl Ethyl Barbiturate, Lilly) and phenobarbital sodium have been used successfully in the treatment of convulsive seizures due to penicillin G.<sup>8,9</sup> Thus, it was of interest to ascertain whether or not Sodium Amytal injected intravenously would also decrease the toxicity of penicillins AT and BT when injected intradurally.

The absorption, distribution, and excretion of injected penicillin, with particular reference to its presence in spinal fluid, have been investigated by several workers.<sup>10-14</sup> In our laboratories, studies were made on the concentrations of penicillins G, AT, and BT in the blood, urine, and spinal fluid of dogs following intradural and intramuscular administration. Since allergic responses to penicillin G have been reported in man,<sup>15,16</sup> and the sensitization of laboratory animals demonstrated,<sup>17-18</sup> similar experiments were undertaken with the penicillins under discussion.

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\*The natural and biosynthetic penicillins differ one from another according to the constituent present in the side chain containing the amide group. Benzylpenicillin (G) may be represented as  $C_6H_5-CH_2-R$ , allylthiomethylpenicillin as  $CH_2=CH-CH_2-S-CH_2-R$ , and n-butylthiomethylpenicillin as  $CH_3-CH_2-CH_2-CH_2-S-CH_2-R$ , where R represents the remainder of the penicillin molecule.

## PROCEDURES

Mice weighing 12 to 14 grams were selected for the intracerebral tests. The tops of their heads were painted with Tincture Merthiolate (Sodium Ethyl Mercuri Thiosalicylate Lilly), and injections made with a 0.5 inch 26 gauge short beveled needle, through the thin parietal bone near the sagittal suture. The compounds to be tested were dissolved in physiologic saline and injected in doses of 0.03 c.c. per mouse. Ten or more mice were used per dose, and the median lethal dose  $\pm$  standard error was computed by the method of Bliss.<sup>19</sup> Intracisternal injections were made on rabbits by means of a short beveled 20 gauge spinal needle. Clear fluid was withdrawn and various concentrations of the compounds in physiologic saline were administered in small volume doses (0.15 to 0.50 c.c.). Cisternal punctures on dogs were made through the space between the occipital bone and the posterior arch of the atlas. After the escape of a few drops of clear fluid, small volume doses in physiologic saline were injected. Intralumbal injections were also made in dogs in the third or fourth intervertebral space. Mice, rabbits, and dogs which died as the result of intradural injection were subjected to necropsy and sections of the brain and spinal cord examined microscopically.

In an attempt to decrease acute intoxication of penicillin in rabbits Sodium Amytal was injected intravenously in a dose of 10 mg. per kilogram immediately before lethal intracisternal injections of penicillin and again after the first convulsion.

Three dogs were injected, one each with a nonconvulsive dose (200 units per kilo gram) of penicillins G, AT and BT respectively by way of the cisterna magna. Cerebrospinal fluid (CSF), blood and urine samples were collected hourly. The paper disc method of Loo and associates<sup>20</sup> for streptomycin adapted to the estimation of penicillin was used in the determination of the antibiotic content in the urine and cerebrospinal fluid. The blood concentrations were tested by a modification of the method described by Rommelkamp.<sup>21</sup> In an effort to find the rate of distribution of the penicillins from the blood into the cerebrospinal fluid, similar tests were made following intramuscular injection.

Sensitization experiments were conducted on guinea pigs. The animals were injected daily for five days by the intraperitoneal route with 500 to 1000 units of the respective penicillins. At the end of twenty one and forty two days they were tested for anaphylaxis by intravascular injection. In addition some of the animals were sacrificed and their isolated uteri tested for sensitivity to the compounds by the method of Scholtz and Dale.<sup>22, 23</sup>

## RESULTS

In contrast with the low toxicity following intravenous or intramuscular injection penicillin G when administered in relatively small doses into nervous tissue causes death. Compared with penicillins AT and BT, penicillin G is the most toxic. The data presented in Table I show that on a weight basis penicillin AT is  $\frac{1}{8}$ , and penicillin BT  $\frac{1}{18}$  as toxic as penicillin G when injected by the intracerebral route in mice. On a unit basis penicillin BT is  $\frac{1}{38}$  as toxic as the natural penicillin.

TABLE I TOXICITY FOLLOWING INTRACEREBRAL INJECTION IN MICE

PENICILLIN COMPOUNDS	UNITS PER MO	NUMBER OF MICE	LD <sub>50</sub> $\pm$ STANDARD ERROR	
			MO/KO	UNITS/KO
G	1,640	110	57 $\pm$ 0.23	9,348 $\pm$ 377
AT	1,660	30	45.08 $\pm$ 3.91	748.2 $\pm$ 6,491
BT	3,330	30	100.74 $\pm$ 6.67	357,000 $\pm$ 22,211

*Streptococcus hemolyticus* O 63 was used in these assays. This organism responds somewhat differently from *Staphylococcus aureus* to the various penicillins. In order to express our findings in comparable terms the blood concentration values presented in Tables III and V have been corrected to correspond to units as determined with *Staphylococcus aureus* 4991.



Intracisternal injections of rabbits (Table II) show much the same relationships in toxicity. The differences, however, when tested by the same method on dogs are not as great. Penicillin AT is  $\frac{1}{6}$ , and penicillin BT  $\frac{1}{10}$ , as toxic as penicillin G.

TABLE II TOXICITY FOLLOWING INTRACISTERNAL INJECTION IN RABBITS AND DOGS

PENICILLIN COMPOUNDS	SPECIES	NUMBER OF ANIMALS	LD <sub>50</sub> ± STANDARD ERROR (MG/KG)
G	Rabbit	15	0.633 ± 0.065
AT		10	5.61 ± 0.65
BT		10	15.60 ± 2.00
G	Dog	10	1.118 ± 0.112
AT		10	6.49 ± 1.20
BT		11	11.50 ± 0.90

The most pronounced pathologic manifestation of the three penicillins following lethal intracisternal injections in mice and intracisternal injections in rabbits was necrosis of some of the ganglion cells of the cerebrum and Purkinje's cells of the cerebellum. The majority of the cells in both regions were normal. In dogs dying as a result of injections into the cisterna magna, much the same phenomena occurred. In sections from the cerebral cortex, basal ganglia, pons, cerebellum, and medulla, some ganglion cells were necrotic. Most necrosis was seen in the hippocampus major.

Sodium Amytal was found to be capable of preventing acute intoxication produced by intracisternal injections of penicillins G and AT in rabbits. The median lethal dose (LD<sub>50</sub>) for penicillin G was raised from 0.635 to 3.85 mg per kilogram when administered in conjunction with Sodium Amytal—a sixfold decrease in toxicity. Because of the small amount of penicillin AT available, its LD<sub>50</sub> with Sodium Amytal was not established. However, four rabbits when injected, respectively, with one to four times the LD<sub>50</sub> of penicillin AT, preceded and followed by the barbiturate, survived. Two rabbits receiving five times the LD<sub>50</sub> of penicillin AT, together with Sodium Amytal, died within thirty-six hours. No change in the LD<sub>50</sub> of penicillin BT occurred following the use of Sodium Amytal, phenobarbital sodium, urethane, or chloral hydrate.

Table III shows the data on the penicillin concentrations in blood, urine, and cerebrospinal fluid following intracisternal injections in dogs. A comparatively rapid clearance of the antibiotic substance from the spinal fluid occurred in each case.

When these compounds are injected by the intralumbar route in dogs, approximately five times as much material is required to cause death. Presumably, this may be due to the greater time required before the penicillin reaches the brain, giving an opportunity for dilution, as well as loss to the systemic circulation. This effect is demonstrated in Table IV.

The rapid transfer of penicillin from the spinal fluid following intracisternal injection in dogs suggested that adequate concentrations (0.03 unit per cubic centimeter or higher) might be obtained in the spinal fluid by intramuscular injection. Following a dose of 200,000 units, an appreciable amount of the antibiotic substance was demonstrated in the spinal fluid, as shown in Table V.

TABLE III ABSORPTION, DISTRIBUTION AND EXCRETION AFTER INTRACISTERNAL INJECTION IN DOGS—500 UNITS PER KILOGRAM

PENICILLIN COMPOUNDS	DOSE IN UNITS	TIME IN HOURS	PENICILLIN CONCENTRATION (UNITS/CC)			URINE COLLECTED (CC)	UNITS EXCRETED IN URINE	PER CENT EXCRETED
			BLOOD	URINE	CSP			
G	6 450	1	0.3	8.20	118.4	116	0.1	40.49
		2	0.16	7.05	37.0	169	839	
		3	0.02	14.80	32.5	38	562	
		4	<0.02	4.45	10.1	40	178	
		5		3.30	11.4	16	53	
		6		1.50	2.9	10	28	
		Total				298	2 612	
AT	6 100	1	0.29	11.30	285	116	1,311	64.6
		2	0.15	7.70	68	199	1 532	
		3	<0.01	26.40	8.8	24	634	
		4		16.60	<3.0	10	315	
		5		7.25		14	101	
		6		4.30		11	47	
		Total				287	3 941	
BT	6 500	1	0.26	2.64	242	274	722	39.72
		2	0.1	6.70	81	138	925	
		3	0.01	18.20	13.8	31	564	
		4	0.0	18.20	<2.8	11	200	
		5	0.03	5.85		10	58	
		6	0.03	3.15		10	31	
		Total				474	2 501	

TABLE IV TOXICITY FOLLOWING INTRALUMBAR INJECTION IN DOGS

PENICILLIN COMPOUNDS	NUMBER OF DOGS	LD <sub>50</sub> ± STANDARD ERROR (MG/KG)
G	10	4.94 ± 0.27
AT	19	38.00 ± 3.20
BT	15	56.20 ± 2.80

TABLE V ABSORPTION, DISTRIBUTION AND EXCRETION IN DOGS AFTER INTRAMUSCULAR INJECTION—200 000 UNITS PER KILOGRAM

PENICILLIN COMPOUNDS	DOSE IN UNITS	TIME IN HOURS	PENICILLIN CONCENTRATION (UNITS/CC)			URINE COLLECTED (CC)	UNITS EXCRETED IN URINE	PER CENT EXCRETED
			BLOOD	URINE	CSP			
G	1 400 000	1	68.48	3.300	1.32	39	105 600	49.49
		2	68.48	1.230	>2.64	126	154 980	
		3	34.24	14.750	0.66	0	132 750	
		4	26.40	21.000	0.66	8	168 000	
		5	27.40	6.800	0.66	13	88 400	
		6	13.70	5.400	0.66	8	43 200	
		Total				201	642 930	
AT	1 240 000	1	370.00	37.750	0.66	0	303 750	62.4
		2	92.40	22.750	0.66	11.5	261 625	
		3	18.48	14.325	0.33	8.5	121 762	
		4	10.36	1.320	0.33	53	69 960	
		5	2.29	2.100	0.16	5	10 500	
		6	1.12	1.100	<0.02	5	6 050	
		Total				92.5	713 647	
BT	1 480 000	1	52.80	5.400	0.08	15	81 000	31.94
		2	57.80	9.90	0.33	26	25 740	
		3	89.32	3.350	0.33	48	160 800	
		4	42.24	10.836	0.33	10.5	113 775	
		5	21.90	22.250	0.17	3	66 750	
		6	21.90	8.250	0.17	3	24 750	
		Total				105.5	472 815	

Not included in the table are the almost negligible results produced by a dose of 20,000 units per kilogram

Eleven of sixteen guinea pigs sensitized to penicillin G showed a definite response on the twenty-first and forty-second days by the Schultz-Dale test. A group of nine showed no effect on the twenty-first day, but in another group of eight, five exhibited a mild anaphylactic reaction to intracardiac or intravenous injection of penicillin G. No response was obtained by either method when penicillin AT or BT was employed as the sensitizing and challenging agent. Nor was there any response to penicillins AT and BT in guinea pigs sensitized to penicillin G.

#### DISCUSSION

It has been demonstrated that penicillins AT and BT are less toxic by intradural injection than penicillin G. However, lethal doses of all three penicillins have the same pathologic effect upon the brain of mice, rabbits, and dogs, namely, production of necrosis of the ganglion cells.

The toxicity of penicillin G has been a limiting factor in its use in intradural therapy. The concomitant use of Sodium Amytal makes possible the administration of considerably larger doses. The median lethal dose of penicillin AT, which is much greater than that of penicillin G, can be increased further by the use of the same barbiturate. In sharp contrast, efforts to increase the  $LD_{50}$  of penicillin BT, the least toxic member of this group, have so far been unsuccessful. However, the toxicity of penicillin BT without Sodium Amytal is so low that the use of penicillin G or AT with Sodium Amytal offers no advantage.

The absorption, distribution, and excretion rates of these compounds in dogs are much the same, depending more on the size of the dose than upon differences in molecular structure. With the small number of dogs used, no significant differences could be ascertained. When relatively small doses are injected intracisternally, the penicillins studied are rapidly absorbed, distributed, and excreted. It is apparent that the barriers between the cerebrospinal fluid and the blood are relatively permeable to penicillins in the direction of spinal fluid to blood, although they hinder greatly the passage of the antibiotics in the direction of blood to spinal fluid.

Enormous intramuscular doses were required to give rise to definite quantities of penicillin in the cerebrospinal fluid. A dose of 20,000 units per kilogram was not sufficient, but ten times this amount resulted in high concentrations in the spinal fluid of all dogs. At no time, however, did the concentration in the cerebrospinal fluid approach that of the blood.

In view of the fact that penicillins AT and BT, in contrast with penicillin G, have shown no sensitization in guinea pigs, the new products may prove clinically more advantageous in allergic individuals. In all probability, penicillins AT and BT will not be contraindicated following penicillin G, because they have shown no cross-sensitization with this substance.

## SUMMARY

1 Allylthiomethylpenicillin (AT) and *n*-butylthiomethylpenicillin (BT) are much less toxic than penicillin G (benzylpenicillin) when applied directly to the central nervous systems of mice, rabbits, and dogs

2 The toxicity of penicillins AT and G in nervous tissue is reduced approximately fivefold by the intravenous administration of Sodium Amytal. The median lethal dose of penicillin BT, the least toxic of the group, is not affected by the use of the same barbiturate

3 Necrosis of the ganglion cells occurs following lethal, intradural doses of each of the three penicillins

4 The diffusion of penicillin appears to be much easier from the cerebrospinal fluid to the blood than in the opposite direction

5 Sensitization has been shown in guinea pigs to penicillin G, but not to penicillin AT or BT

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## LABORATORY METHODS

### A METHOD FOR THE DETERMINATION OF FIBRIN APPEARANCE TIME

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ADEQUATE methods for control of administration of anticoagulants are of especial importance in light of the increased application of anticoagulant therapy in thrombophlebitis and in postoperative vascular surgical problems. Diemmler therapy is regulated adequately by the determination of prothrombin time, a laboratory procedure; heparin administration on the other hand, is usually controlled according to the blood coagulation time. The blood coagulation time determinations are routinely a bedside and not a laboratory procedure, and all too frequently the results are incorrect.

Among the sources of error in the Lee White<sup>1</sup> and the de Tikats methods or their modifications are the agitation involved in venipuncture and transfer of specimens, the variation in agitation of the specimens and especially the non-uniform sensing of the end point by different observers. The scrupulous cleanliness and uniformity of glassware necessary and the difficulty of temperature control also combine to make the methods cumbersome and of doubtful application to the control of heparin therapy. On the whole these methods require an attention to detail difficult to accomplish on the wards.

In this report we introduce a new criterion for the control of heparin therapy which is not in the true sense a blood clotting method but rather a measurement of the fibrin appearance time. The technical procedure follows.

A capillary tube varying from 1 to 2 mm in diameter and of approximately 5 cm in length is the basic piece of equipment. The tubes are cleaned by dichromate solution followed by thorough rinsing in distilled water. Prior to the test, a 100 cc. Veronal saline buffer solution† (pH 7.4) containing 400 mg. fibrinogen‡ is made up. This solution has been found to remain relatively stable at room temperature for at least twenty-four hours. A small quantity of the fibrinogen solution is drawn up into the capillary tube and any excess solution remaining at the tip of the tube is removed by touching the tip to any clean absorbent material. The finger tip is cleansed thoroughly with alcohol, allowed to dry, and jabbed with a sharp instrument so that a drop of blood will appear immediately without the necessity of milking the finger. The tip of the capillary tube is touched to the drop of freshly drawn blood and a quantity roughly equal to that of the fibrinogen solution already present is allowed to rise into the tube.

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†Four hundred milliliters of 0.1M sodium diethyl barbiturate mixed with 88 ml. 0.1M HCl and diluted to 1,000 cc. with oxalated saline (0.5 per cent).

‡Supplies of purified bovine fibrinogen were made available by Parke Davis & Company, Detroit, Mich. and by The Armour Laboratories, Chicago, Ill.

by capillarity. The end is sealed carefully with petroleum jelly, and the capillary tube is placed in a constant temperature bath at  $37.5^{\circ}\text{C}$ . A bright light placed behind the capillary tube enhances the visibility of the fibrin formation. The end point is read when the first cloudy haze appears at the blood-fibrinogen solution interface, and is not to be confused with the plasma layer formed when the blood cells sediment. The time of the test extends from the moment blood is drawn from the finger to the moment the cloud at the interface appears.

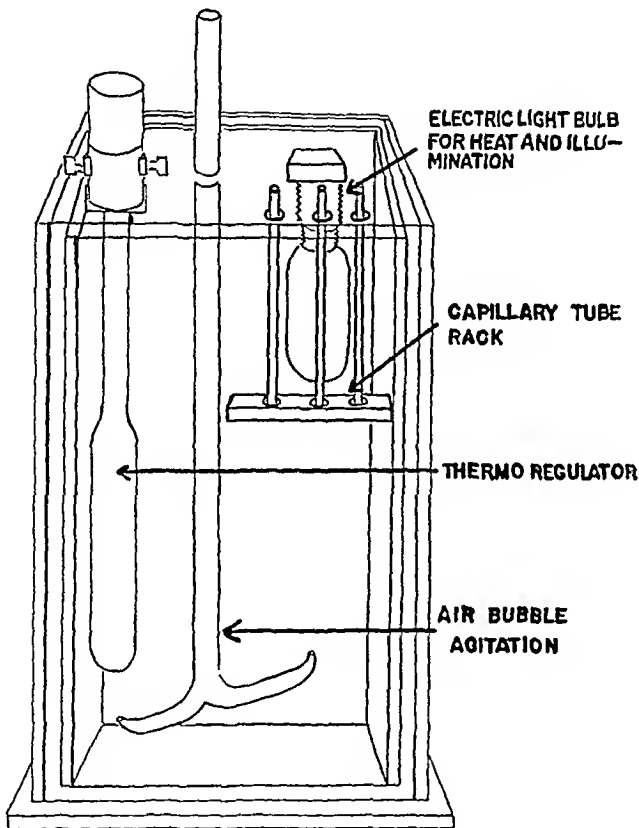


Fig 1—Diagram of the Lucite water bath used to maintain a constant temperature of the capillary tubes

The theoretic basis of the test is the accepted concept that fibrinogen in solution is converted to fibrin by the action of thrombin formed in the blood. Since thromboplastin in the presence of calcium ion effects the conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin, it would appear that if a clear solution of fibrinogen were to be brought into contact with whole unclotted blood, thrombin activation by blood and tissue thromboplastin would be reflected immediately by fibrin conversion in the portion of the fibrinogen solution contiguous to the whole blood. The arrangement which appeared to offer the greatest degree of simplicity was that of layering blood drawn into a capillary tube with a fibrinogen solution. Through observation of such

an arrangement it can be seen that a definite area of fibrin forms in the clear fibrinogen solution just above the blood fibrinogen interface. Using a capillary tube provides an additional advantage in that the rate of diffusion of what is presumed to be thrombin is held relatively constant. It is reasonable to suppose that the conversion of fibrinogen to fibrin at the interface goes on simultaneously with the conversion of fibrinogen to fibrin in the fresh whole blood below. The immediately apparent advantages are that the fibrin formation goes on in a solution which is perfectly clear and not obscured by the whole blood, that agitation is minimal, and that observation can be carried out with ease in a constant temperature bath.

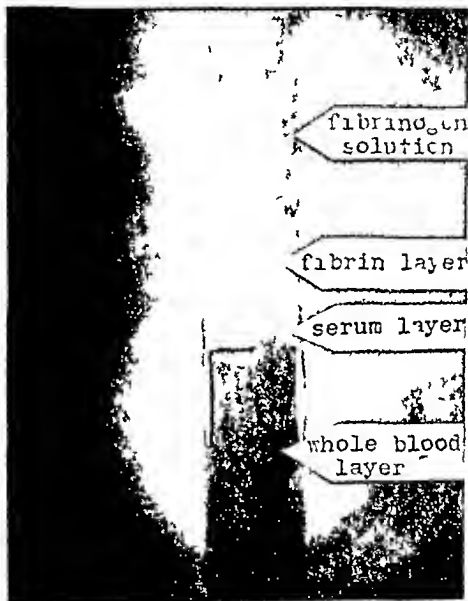


Fig. 2—Photograph of the end point in the capillary fibrinogen method. The source of light is behind the capillary tube.

The simplicity of sampling finger tip blood employed by the de Takats method is favored, although it is well known that blood obtained from finger tip sampling exhibits greater thromboplastic activity than that derived from venipuncture, however. In the data that are to be presented it appears that the summation of tissue and blood thromboplastic activity bears a direct relation to the quantity of heparin given.

A 400 mg per cent solution of fibrinogen was chosen because it approximates the upper limits of normal blood fibrinogen concentration. Various solu-



tions containing from 100 to 800 mg per cent fibrinogen were tried without any appreciable difference in fibrin appearance time or in the visual density of the fibrin formation. Veronal buffer was used with a pH of 7.4 since the fibrin formed was slightly more opaque than that seen with fibrinogen in physiologic saline. Capillary tubes with bores varying from 0.5 to 2.0 mm were used without introduction of any appreciable error.

TABLE I COMPARISON OF CLOTTING TIME DETERMINATIONS MADE SIMULTANEOUSLY BY THE LEE WHITE AND FIBRIN APPEARANCE METHODS AT RANDOM INTERVALS ON INDIVIDUALS RECEIVING HEPARIN

INDIVIDUAL	CAPILLARY FIBRINOGEN METHOD (MIN)	LEE WHITE METHOD (MIN)
1	10	33
	9	20
	8	22
2	13	40
	8	17
	8	16
	12	29
	14	45
3	12	29
	8	16
	10	18
	12	29
	7	17
4	8	21
	11	31
	10	18
	10	23
	6	5
5	7	12
	9	20
	7	20
	10	35
	17	54
6	7	13
	6	16

TABLE II COMPARISON OF CLOTTING TIME DETERMINATIONS MADE SIMULTANEOUSLY IN TRIPLICATE BY THE LEE WHITE METHOD AND FIBRIN APPEARANCE TIME METHOD ON A SUBJECT WHO RECEIVED 20 MG HEPARIN INTRAVENOUSLY

	LEE WHITE METHOD (ROOM TEMPERATURE APPROX 25° C) (MIN)	FIBRIN APPEARANCE TIME METHOD (37½° C) (MIN)
Control	27*	6.5
	30	6.5
	30	6.5
15 min post heparin	60	15.0
	48	15.5
	60	15.5
35 min post heparin	39	9.5
	37	9.5
	33	9.5
60 min post heparin	19	2.0
	21	2.0
	21	2.0
270 min post heparin		6.0

\*The control values here are higher than those found in routine clotting determinations on normal individuals for two reasons. Determinations were made in test tubes with a diameter of 12 mm rather than the routine 8 mm and were conducted at a temperature of 25° C rather than 37.5° C. The normal time spread at 37.5° C in 8 mm diameter tubes is eight to fifteen minutes.

A large series of presumably normal individuals were tested and it was found that the spread of normal fibrin appearance time fell within five to seven minutes. When repeated tests were run with a single individual the variation was approximately thirty seconds.

A series of patients receiving heparin was tested simultaneously with the fibrin appearance time determination and the Lee White coagulation time

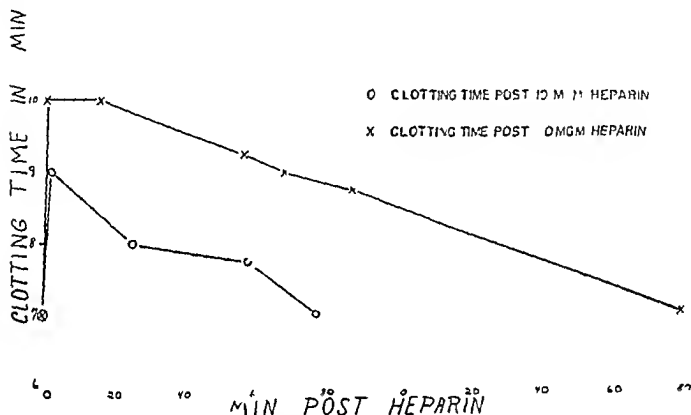


Fig 3—Heparin tolerance test

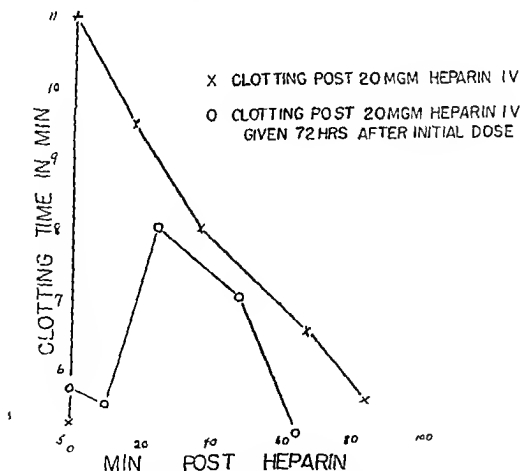


Fig 4—Heparin tolerance test.

method There was a rough correlation between the two times but a constant relationship was not apparent (Table I)

In an additional attempt to establish a strict correlation, 20 mg of heparin (Liquaemin) were given intravenously to a subject, and at predetermined intervals triplicate tests were run both by the Lee-White coagulation time method and the fibrin appearance time method Again the times were roughly parallel, but there was no precise constant (Table II)

Our attention was focused on the alteration of fibrin appearance time by heparin administration Fig 3 presents the findings obtained when 10 mg of heparin (Liquaemin) were given intravenously to a normal individual Three hours following the initial dose of 10 mg, 20 mg were given intravenously The

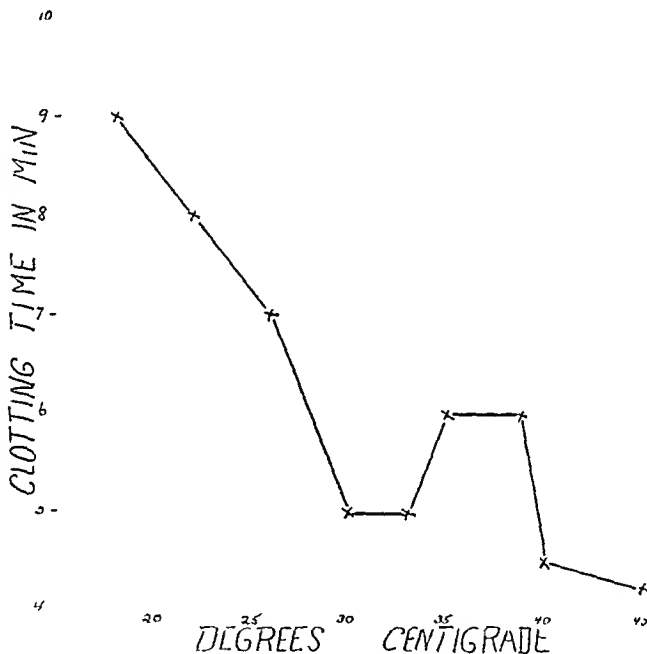


Fig 5 —Effect of temperature on clotting time

prolongation of the fibrin appearance time was immediate in both instances, and subsequent drops in the fibrin appearance time progressed in a linear and parallel fashion as the effect of the drug diminished Fig 4 depicts a similar test in another normal subject, substantiating the fact that among presumably normal individuals there is considerable difference of response to heparin In the second subject the initial prolongation of the fibrin appearance time was much greater and the subsequent fall-off was more rapid

It was found that if heparin is given after a lapse of several days, the individual's response may be markedly altered (Fig 4) Seventy-two hours following the initial intravenous heparin dosage, 20 mg of Liquaemin were given intravenously The individual's response had been markedly altered Height and duration of the curve had diminished

In order to determine the importance of temperature control repeated fibrin appearance time determinations were made on the same individual and were carried out in a water bath in which temperature was carried from 20 to 40° C. (Fig. 5). At the lower temperatures the expected prolongation of the fibrin appearance time occurred.<sup>3</sup> At 30° C. the time reached its shortest interval but as body temperature was approached it again rose. Just above normal body temperature it again fell back to low levels leveling off at 40 to 45° C. What significance this observation may have is unknown but it does give rise to speculation as to whether the same phenomenon occurs *in vivo* as *in vitro*.

#### SUMMARY

A method for the determination of fibrin formation time based on the diffusion of thrombin into a fibrinogen solution is described. The method appears to reflect more accurately heparin activity *in vivo* than the conventional clotting time determinations. The method lends itself to heparin studies on small animals more readily than the Lee White method. Data presented suggest the possibility of the development of an increased tolerance for heparin on periodically repeated intravenous dosage. It appears that fibrin appearance time varies sharply with *in vitro* temperatures that are within physiologic limits.

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## A SIMPLE MIXING AND SHAKING APPARATUS

THOMAS F. FRAWLEY, M.D.,\* AND CHARLES W. BISHOP, PH.D.  
BUFFALO, N. Y.

THE accompanying photograph (Fig. 1) illustrates a simple, rugged, portable, inexpensive mixing and shaking apparatus. The development of this apparatus was an outgrowth of investigations into ketone body metabolism involving the use of the micromethod of Greenberg and Lester for the determination of acetone and ketone bodies†. However, its usefulness has since been extended to several other laboratory methods. These applications and the potential utility of such an apparatus in a laboratory have prompted this description.

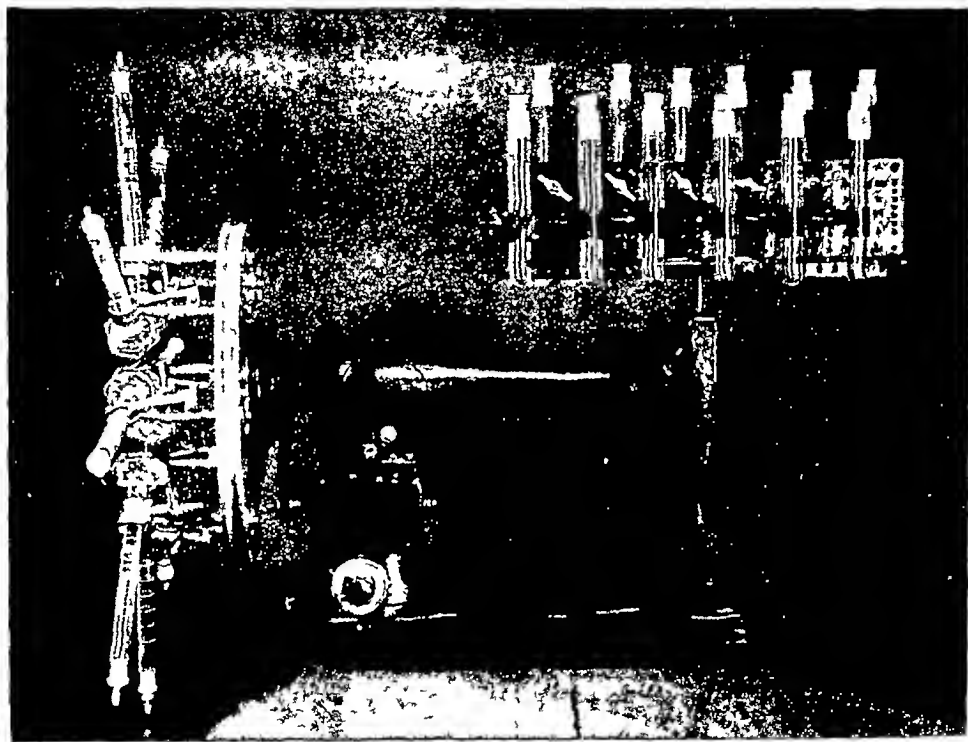


Fig. 1

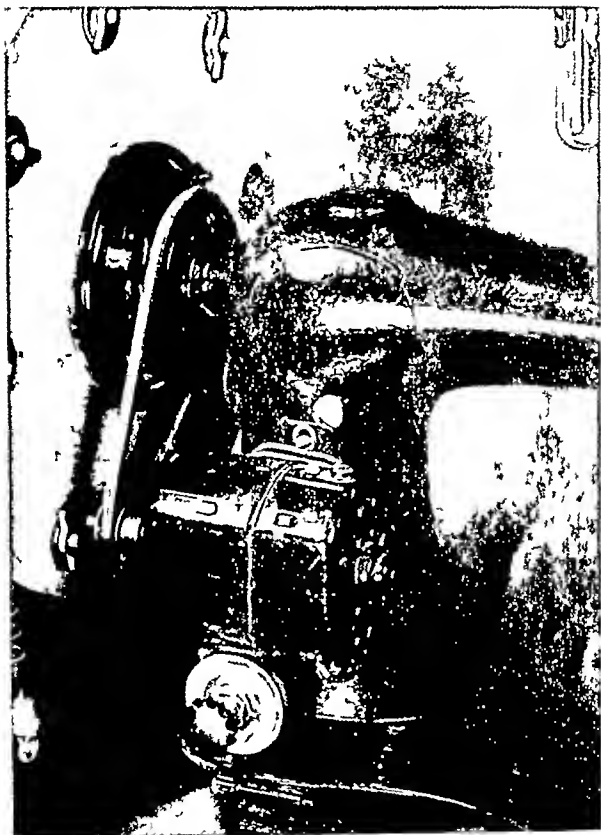
The apparatus consists of a sewing machine which has been modified to give both a vertical type of shaking motion and a rotary type of mixing motion. Several containers can be managed simultaneously in only one or in both of these motions. The adaptation of a sewing machine to give mixing by the technique of inversion was heretofore unknown to the authors.

From the Medical Research Laboratory of the Buffalo General Hospital

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†Greenberg, L. A. and Lester, D. Micromethod for Determination of Acetone and Ketone Bodies. *J. Biol. Chem.* 154: 177, 1944



Fig

## A DESCRIPTION OF THE APPARATUS

An old foot operated sewing assembly was removed from its base and fitted to a small wooden platform so that it could be placed on a laboratory bench. A circular wooden disk, 10.2 inches in diameter and 0.7 inches thick was then bolted to the drive wheel. The disk contained eight equally spaced small, utility clamps from which the clamp holders had been removed. The clamps were located one inch from the periphery of the disk and fastened in place with the customary locking brass wing nut accompanying clamps of this type. The disk was in turn rotated by a small motor of the type commonly used for converting sewing machines (Fig. 2).

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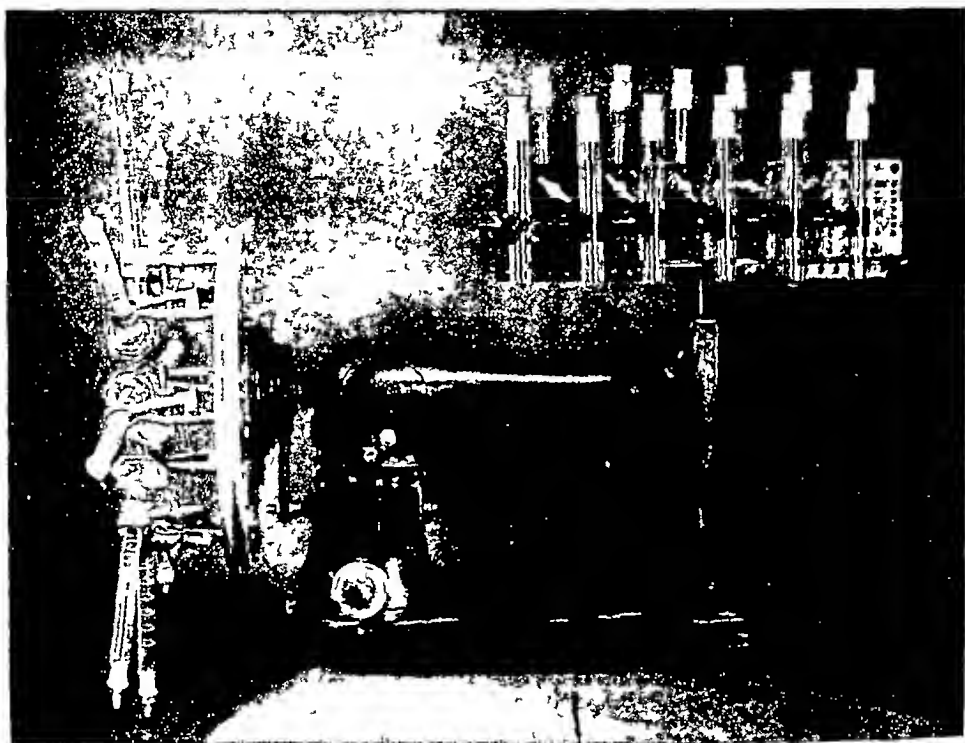


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# AN ELECTRONIC APPARATUS FOR RECORDING BLOOD PRESSURE

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MANY biologic assays and pharmacologic investigations depend on the measurement of the mean arterial blood pressure. This is commonly accomplished by the use of the Ludwig mercury manometer writing on soot covered paper. The soot covered paper is messy, difficult to label and susceptible to cracking or sticking during storage depending on the type of fixative used. Although the disadvantages of the soot and stylus procedure can be partly overcome by the use of a writing point using ink, this device requires constant attention to keep the ink reservoir filled and the recording paper must be run fairly rapidly to avoid having the ink smear and run.

With sufficient patience and experience satisfactory records can be obtained with either of these techniques but the mercury manometer suffers the additional faults that a fairly large hole must be made in a major artery that some blood loss is always involved and that an anticoagulant must be used. These factors make the use of unanesthetized animals or small animals difficult. Some of these defects can be overcome by the use of the rubber diaphragm manometer but the device is nonlinear in response and possesses the same recording defects.

The Hamilton<sup>1</sup> and Wiggers' optical recording manometers are precision instruments that give a true record of systolic and diastolic blood pressure from which the mean arterial pressure can be calculated. Hypodermic needles are used for cannulas and blood pressure can be measured without the necessity of making large incisions. These devices are difficult to set up and to maintain in operation, must be used in partial darkness, and since they record on photographic paper there is a considerable time lag between the administration of a drug and observation of the results. Recently Braunstein and co workers<sup>2</sup> have suggested the use of strain gage manometers recording through a string type electrocardiograph, but this also suffers the difficulties inherent in any method of photographic recording.

All of these difficulties can be overcome by the use of the following apparatus, the components of which are commercially available. A Lambert Wood<sup>3</sup> strain gage manometer\* has its two input terminals connected to a 75 volt A battery,† and its output terminals connected to a General Electric photoelectric type recording microammeter‡. By reducing the size of the plastic head on the manometer and using a hypodermic needle adapter instead of a tubing connection, it is possible to mount the manometer directly on the animal. This has obvious advantages when it is desired to expose the animal to artificial changes

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Various models and pressure ranges available from Statham Laboratories Los Angeles Calif. The illustration shows a modified P8 2 350

\*Burgess G 5 Burgess Battery Company Freeport Ill

†Photoelectric Recording Microammeter Model 8CE1DJ15 from General Electric Company Schenectady N. Y. The 8CE1DJ11 has also given good results



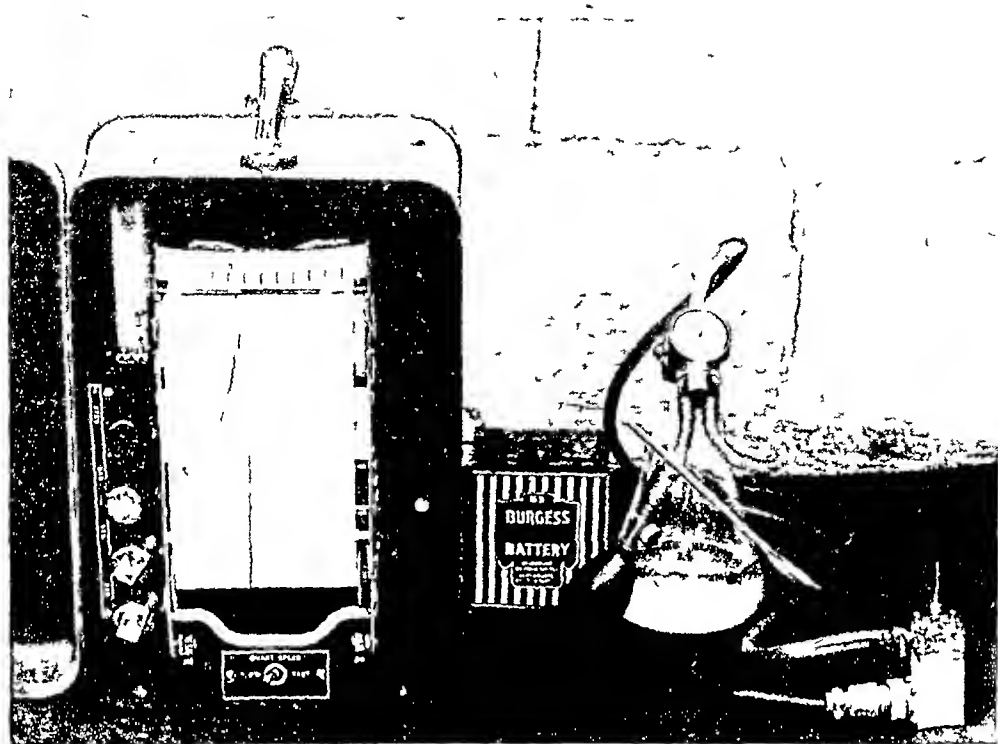


Fig 1—Left to right recording microammeter battery for strain-gage pressure bottle containing heparinized saline for washing out manometer and modified Lambert-Wood strain-gage manometer. The hypodermic needle cannula is coated with General Electric Dri-film 9987 & silicone resin to lower the incidence of clotting at the tip

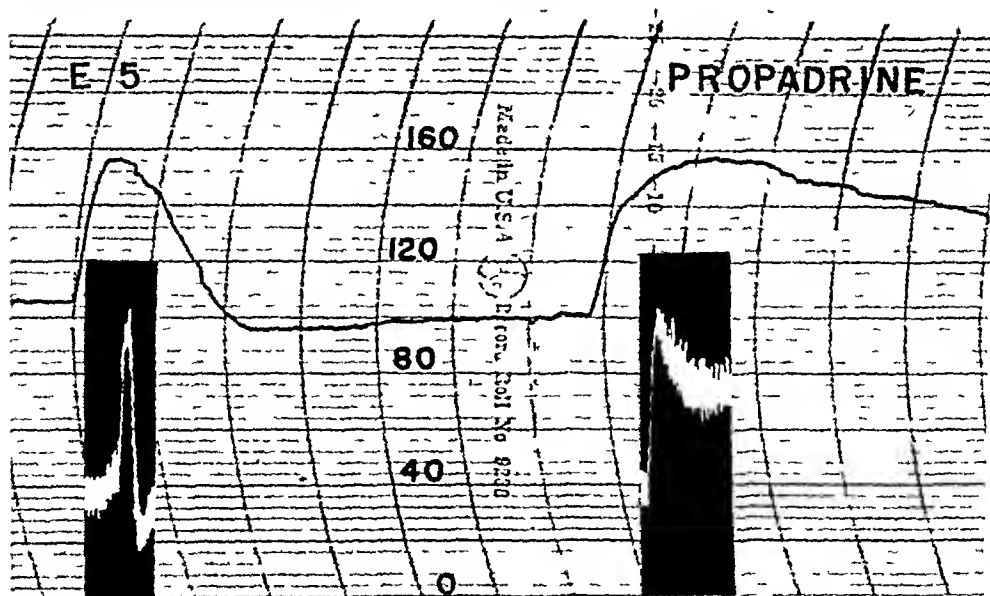


Fig 2—Typical record Dog (11 kilograms male 320 mg sodium barbitol per kilogram intraperitoneally) Strain gage manometer in left carotid artery mercury manometer connected to right carotid artery. Five micrograms of epinephrine (base) per kilogram given at E 5, and 1 mg Propadrine hydrochloride per kilogram given at PROPADRINE. Time vertical lines minutes pressure horizontal lines 4 mm Hg units. Sections of the mercury manometer record run only one-fifth as fast, have been superimposed for purposes of comparison.

of environment as only the electrical connection to the recorder need be run outside of the experimental chamber. Since very little volume exchange occurs with this type of manometer, no blood loss nor introduction of large amounts of anticoagulant solution need occur, and unanesthetized animals can be used and pressure measured as long or as frequently as necessary. The device has been used to measure the blood pressure of rats, rabbits, guinea pigs, cats, and dogs. With suitable precautions as to sterility of the cannula and solution it could be used in man.

The recording microammeter is simple to operate, ruggedly constructed and portable. The ink written record is always visible and annotations can be made on it in ink or pencil at any time. It is not subject to destruction by folding, warmth, or scratching as are some of the electrolytic and plastic recording papers, and it can be easily mounted with glue or rubber cement. The rolls are available with various rulings or plain. Various gear trains are available so that almost any desired rate of movement of the recording paper can be obtained. By choosing from the many basic instrument movements available and matching with the strain gage sensitivity, almost any desired deflection sensitivity can be obtained. If necessary, the sensitivity can be increased by using a higher voltage battery or decreased by the use of a low ohmage variable resistance between the manometer and recorder. The electronic circuit is slow enough that damping of the systolic diastolic pressure to an integrated tracing of the mean arterial blood pressure occurs, and the minor fluctuations seen with the mercury manometer are absent.

#### SUMMARY

The disadvantages inherent in the common methods of the measurement of mean arterial blood pressure can be overcome by the use of a Lambert Wood strain gage manometer and a portable recording microammeter.

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# AN OPTICALLY RECORDING BUBBLE FLOW METER ADAPTED FOR MEASUREMENT OF RENAL BLOOD FLOW

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THE principle of measurement of the mean rate of blood flow by timing the passage of a small air bubble introduced into the blood stream of an organ was first used by Soskin, Priest, and Schutz<sup>1</sup>. Blood leaving the femoral vein of a dog passed through an external circuit of glass tubing of small bore. Knowing the exact volume from the point of entrance to the point of exit of the tubing and the time of passage of the bubble between these two points permitted calculation of volumetric flow. Dumke and Schmidt<sup>2</sup> have more recently employed a modification of this principle for estimation of cerebral blood flow by measuring arterial inflow, and the same apparatus has been used for coronary arterial inflow measurement<sup>3</sup>. Both devices have required direct visual observation for timing bubble passage. However, when measurement of renal blood flow is to be made in dogs, rates up to 200 cc per minute are not uncommon and time of bubble passage is correspondingly brief. To insure complete accuracy a photoelectric cell was incorporated into the present modification to measure the time of bubble passage. This has the additional advantage that it can be used to actuate an optically recording galvanometer so that flow can be registered simultaneously with blood pressure measurements on the photokymograph.

*Description of Apparatus*—(Numbers in parentheses refer to corresponding parts of the apparatus pictured in Fig 1). The main circuit consists of Lucite (Plexiglas) coiled tubing of about 3 mm internal diameter and 60 cm length. Blood enters and leaves the tubing through a Lucite block through which the bore of the tubing is continuous (10). A metal plate containing two slots directly overlying the continued bore of the tubing covers the Lucite block. Through these slots light from a 6 volt auto lamp (9) is transmitted to an underlying photoelectric cell (11). Air from a reservoir under pressure somewhat higher than carotid blood pressure is introduced at (6) via a three-way stopcock (5) into a 2 cc syringe (2), and from this volumes of about 0.3 cc are injected into the inflow limb of the meter. Passage of this bubble under the "entering" and "exit" slots suddenly transmits light through the column of blood to the photocell. This impulse is amplified by a G E Victor electrocardiograph, causing the mirror of an auxiliary galvanometer of the torsion-string type to register a deflection of a light beam (see record, lower part of

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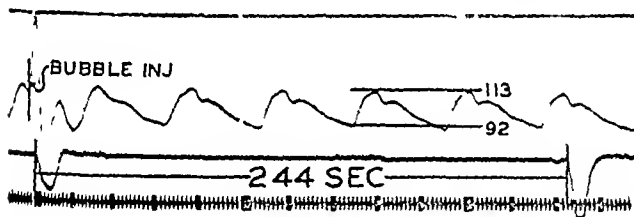
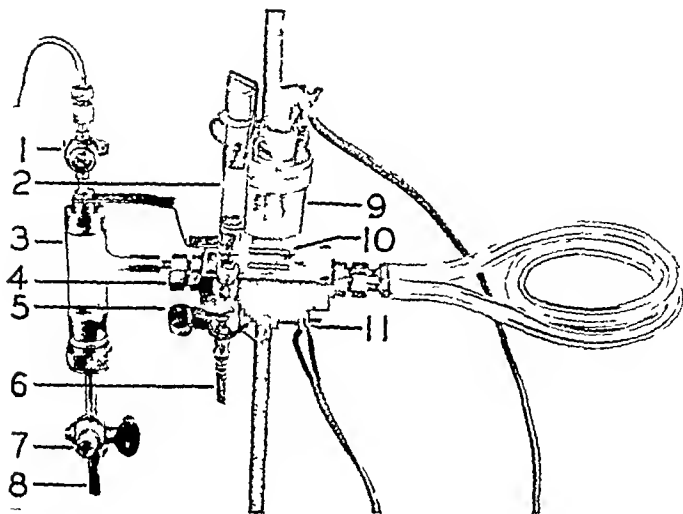


Fig 1—Top optically recording bubble flow meter utilizing photoelectric cell to record time of bubble passage. The numerals refer to various parts which are described in the text. Bottom optical record showing (from above downward) manometer base line renal arterial pressure and galvanometer record showing characteristic deflections as bubble passes entering and exit slots of meter. Volume of the flow meter circuit between slots is 6 cc hence observed flow is  $6 / .44 \times 60 = 147$  c.c. per min. corrected flow (from Fig 2) is 130 c.c. per minute.

Fig 1) The air bubble is then captured in a trap made from large diameter glass tubing with a side arm (3). As air accumulates it is released through a stopcock (1).

Measurement of the rate of arterial inflow is provided for by cannulation of a carotid artery and connecting with lead tubing to the inflow coupling of the meter (4). The blood leaves the meter at (8) which connects directly to a metal "L" cannula (not shown in the figure) suitable for cannulation of the renal artery. At this point another coupling (7) permits connection to an optical manometer of the Gregg type for registration of renal arterial pressure.

No provision has been made for constant temperature regulation of the flow meter. At normal rates of renal blood flow, temperature drop across the meter is hardly perceptible. Indeed, for a flow of 42 c.c. per minute, a temperature drop of only  $0.25^{\circ}\text{C}$  was noted with ambient temperature of  $23^{\circ}\text{C}$ . Any cooling of the animal can be prevented by use of a warming board.

*Calibration of the Instrument*—To calculate volumetric flow, the exact volume of the flow meter from mid-point to mid-point of the "entering" and "exit" slots must be known. Time of passage is obtained from the electrical

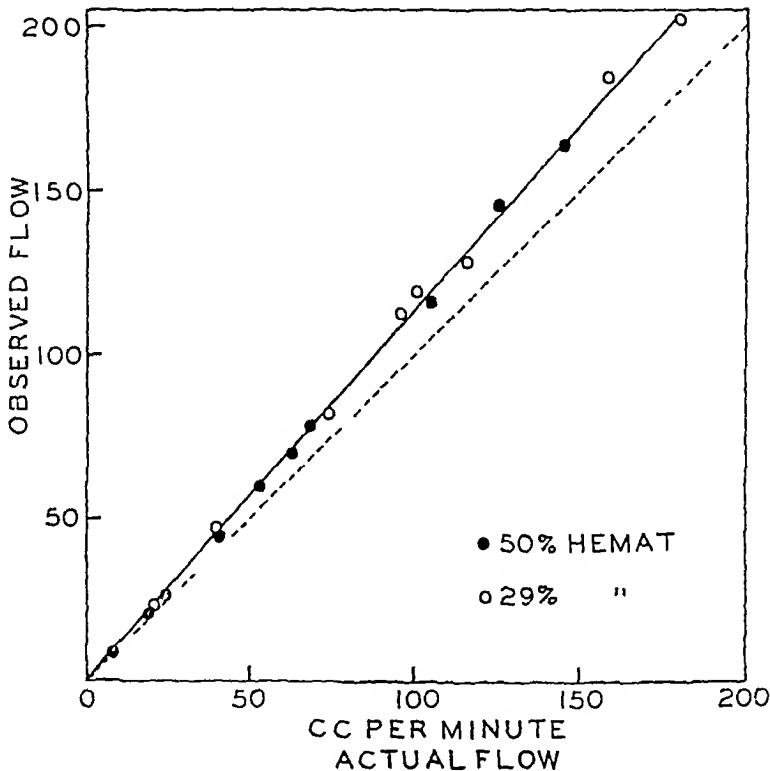


Fig. 2—Calibration graph showing the relationship of observed flow to direct volumetric flow. The dotted line indicates perfect correlation.

timing device of the photokymograph. It was found that the air bubble traveled somewhat faster than the total column of blood, making the observed flow faster than the simultaneously measured volumetric outflow. This is a linear relationship directly proportional to the rate of flow and the total length of the flow-circuit. Hence a calibration curve of the type illustrated in Fig. 2 must be constructed by making simultaneous direct flow determinations with bubble flow at different rates. Hematocrit concentration does not appear to significantly influence this relationship as shown in Fig. 2.

*The Effect of the Bubble Meter on Renal Arterial Pressure and Pulse*—The blood meets resistance to flow in the meter circuit, causing mean renal arterial perfusion pressure to be less than mean systemic arterial pressure. The

pressure drop in the meter used here is 16 mm Hg when blood flows through the meter at a rate of 100 cc per minute (average hematocrit reading 38 per cent). Pulse pressure averaged 18 mm Hg for a number of observations at an average heart rate of 160 per minute. Due to the damping effect of the meter there is a change in normal carotid pulse contour resulting in a more gradual rise of the pulse and a considerable damping of the incisura (Fig 1). As air accumulates in the bubble trap the change in contour becomes more marked, finally giving a triangular contour with no incisura accompanied by further reduction in pulse pressure.

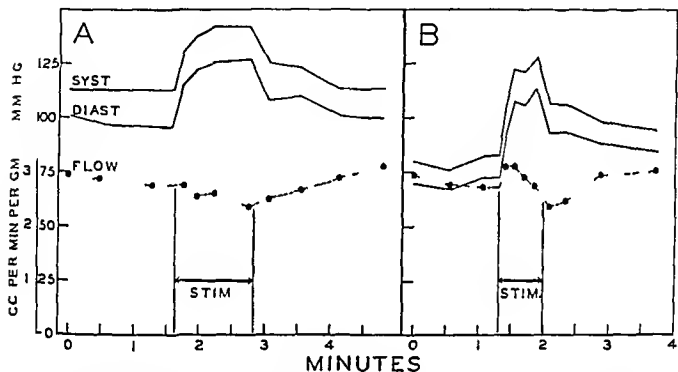


Fig 3—Representative experiments in two animals showing reflex vasomotor effects on kidney flow. A sciatic stimulation (traction). B central vagal stimulation (Electrodynic stimulator 1.0 volts @ 60 per second).

**Preparation of Animal**—Dogs anesthetized with 30 mg per kilogram of body weight of pentobarbital sodium administered intravenously, are used. Utilization of the arterial inflow principle necessitates exposure of the left renal artery by a dorsal retroperitoneal approach. About 1 cm of the renal artery close to the abdominal aorta is dissected free of nerve fibers, if this is done carefully they need not be interrupted. Before cannulation of the artery, the animal is heparinized with 4 mg per kilogram of body weight priming dose, and a total of 5 mg of heparin is given every half hour thereafter. Cannulation requires first ligation of the artery close to the aorta before incising. The renal cannula is then introduced toward the kidney and secured with a second ligature. Total time of this procedure should be less than one minute.

**Representative Results**—From various observations made on a number of animals, only two illustrative experiments demonstrating reflex effects on flow are presented herewith (Fig 3). A shows decreased flow accompanying a pressor response following central sciatic nerve stimulation in one animal, and B shows a phase of reduced flow accompanying a pressor response during

strong central vagal stimulation Since flow is reduced while arterial pressure is elevated, active reflex vasoconstriction must be occurring in the kidney This proves the intactness of the renal nerve supply

#### CONCLUSIONS

Advantages of the optically recording bubble flow meter are (a) it records mean flow, and readings can be very rapidly repeated, e g, every five seconds, (b) moderate amounts of heparin can be used, for clotting is minimal on the nonwetable surface of the Lueite tubing Furthermore, small amounts of fibrin formation should not change the calibration of the instrument appreciably This is not the case with other currently used optical flow meters such as the rotameter With this, anticoagulant dyes such as pontamine fast pink and chlorazol fast pink are required as an adjunct to heparin These dyes are not without their effect on renal blood flow (unpublished observations)

The disadvantage of the present device is that there is appreciable loss of perfusion pressure through the meter and some damping of the pulse pressure Observations here indicate that while reduction in pulse pressure does not influence renal blood flow, decrease in mean perfusion pressure will obviously reduce flow to some degree Accordingly, the best use of the instrument is for measurement of changes from control value resulting from neurogenic or humoral and drug action, particularly when experimental changes are rapid

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## HEMOPHILIA LIKE DISEASE IN WOMEN

### REPORT OF TWO CASES

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**W**HETHER or not hemophilia can occur in women has never been conclusively determined. Hemophilia is a disease characterized by delayed clotting of the blood, is transmitted through women and occurs exclusively in men. However, a few cases which clinically resemble hemophilia in women have been reported. We have recently encountered two such patients.

**CASE 1**—A woman aged 40 was first seen on Jan. 31, 1945 with the complaint of sudden hemorrhage into the base of her tongue that interfered with eating and caused pain and difficulty upon swallowing. She had first noted large hemorrhages into the subcutaneous tissue of her right arm with some swelling and pain in August 1944. These multiple areas of ecchymoses had appeared several days subsequent to a bee sting on her right hand which resulted in severe local redness and swelling. The subcutaneous hemorrhages had continued and one week later had spread to involve the left arm in a similar fashion. She had noted hematuria on one or two occasions and some bleeding from the mouth. The episodes of hemorrhage continued with variable frequency involving various portions of the body.

The patient's father had died at the age of 77 of arteriosclerosis and her mother was living and well at the age of 85. Two brothers aged 62 and 64 were alive and well and no siblings were deceased. There was no family history of abnormal bleeding or bruising for at least two generations. The past medical history was negative except for scarlet fever at the age of 24 which left no sequelae. The patient had had one miscarriage followed by five normal births, the last being in May 1944, without abnormal bleeding. Her menstrual periods had always been regular every twenty-eight days, lasting five days without excessive flow.

Physical examination was entirely negative except for many large subcutaneous hemorrhages over the entire body. The laboratory findings are summarized in Table II. Sternal puncture disclosed an erythroblastic marrow but was otherwise negative.

From January 1945 until April 1945, the patient was given six intravenous injections of plasma by her own physician. She thought she was much improved during this period. In April she sustained a large bruise on the forearm following trauma but the spontaneous hemorrhage in general was much less frequent. Hemorrhagic studies made in January 1946, are summarized in Table II. In March she delivered a normal child and showed no tendency to abnormal bleeding. In June following extraction of a tooth, continuous oozing was present for two days and her entire jaw became "black" for a few days then returned to normal. She was observed again in August with no evidence of bleeding or bruises.

From the Division of Internal Medicine, the Cleveland Clinic and the Frank E. Bunts Educational Institute.

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TABLE I COMPARISON OF PATIENT'S BLOOD WITH THAT OF NORMAL AND HEMOPHILIC SUBJECTS

2 CC WHOLE BLOOD		0.1 CC PLASMA		COAGULATION TIME
N	+	O	=	9 min
P	+	O	=	130 min
N	+	P	=	9 min
P	+	N	=	30 min
H	+	O	=	57 min
H	+	N	=	12 min
H	+	P	=	57 min

N normal patient H hemophilic patient P patient

TABLE II

	JANUARY, 1945	JANUARY, 1946	AUGUST, 1946
Tourniquet test	Neg	Neg	Neg
Platelets	620,000	380,000	370,000
Coagulation time (4 test tube method)	1 hr 56 min	19 min	15 min
Coagulation time Recalcified plasma			
Slow	21 min	3 min	3 min 20 sec
Fast	29 min	4 min 15 sec	6 min 30 sec
Bleeding time	3 min	6 min	6 min
Clot retraction	Normal	Normal	Normal
Prothrombin time (Quick)	15 sec (normal 15 sec)		16 sec
Fibrinogen	490 mg /100 ml		
Calcium	9.4 mg 100 ml		
Wassermann	Neg		
RBC	3,100,000	4,410,000	4,650,000
WBC	7,000	5,100	4,100
Hgb (Haden Hauser)	9.3 Gm	12 Gm	12.5 Gm

CASE 2—A woman, aged 33, was admitted on Feb 14, 1944. In January she had noted some swelling and tenderness of one of the finger joints, which was interpreted as a mild arthritis at the time. She denied any traumatic factors. Later there had been evidence of bleeding in the area of the joint, followed by a similar swelling and tenderness of the right wrist with hemorrhage extending up the right arm. There was subsequent involvement of the left ankle and left leg, with large hemorrhagic areas over the entire left leg and foot. During this period the patient had epistaxis on several occasions from the right nostril only, usually occurring in the early morning or associated with reclining, and persisting from five to forty minutes. The flow was by drop rather than by steady stream and had never been difficult to control. Hematuria had been noted for one week in January, but this had cleared spontaneously and had not recurred. Associated with the appearance of ecchymoses and bleeding into the joints, the patient had noticed pronounced malaise and physical weakness and had become unable to carry on her household duties.

Family history was negative, and careful questioning did not reveal evidence of any familial hemorrhagic tendency on either the maternal or paternal side. There were nine maternal and eight paternal siblings and two sisters of the patient, both of whom were born deaf but were otherwise normal. The past medical history was entirely normal. On Jan 19, 1943, she had delivered a normal baby boy after full term pregnancy with no unusual loss of blood. There had never been any abnormality in the menses.

Physical examination was entirely negative except for ecchymotic areas about the right elbow, inside the right and left thighs, and on the left ankle. There were no manifestations of joint involvement. No petechiae were present.

In March, 1944 the patient was given 250 ml of lyophilized plasma by intravenous infusion. The coagulation time was reduced from two hours and fifteen minutes to one

hour and thirty minutes. This procedure was repeated a few days later and no reduction in the coagulation time was observed. When he was last seen at the Clinic in September 1944, the coagulation time was only one hour and forty five minutes. A telephone conversation in August, 1946 revealed that he was continuing to have ecchymoses and minor joint symptoms. The laboratory data are summarized in Table III.

TABLE III

	FEBRUARY 1944	MARCH, 1944	AUGUST, 1944	SEPTEMBER 1944
Tourniquet test	Neg.			
Platelets	430,000	300,000		
Coagulation time (4 test tube method)	1 hr 45 min	1 hr 15 min	1 hr 50 min	1 hr 45 min
Coagulation time Recliff's plasma				
Slow	10 min	12 min		
Fast	25 min	21 min		
Bleeding time	1 min	1 min		
Cloth retraction	Normal	Normal		
Prothrombin time	19 sec		17 sec (normal 15 sec)	
Calcium	10 mg/100 ml			
Wassermann	Neg.			
RBC	4,940,000	5,130,000	4,900,000	
WBC	7,300	6,200	5,300	
Hgb (Haden-Hauscr)	12.5 Gm	13 Gm	13.5 Gm	

## DISCUSSION

In the past sixteen years at the Cleveland Clinic this type of hemorrhagic diathesis occurring in female patients has been observed on only two occasions. It is undoubtedly a rare condition. Nevertheless we believe that this hemorrhagic problem occurs more frequently than is realized but remains undiagnosed. It is interesting to speculate as to whether or not such women bleeders have the same inherent defect in the blood as that present in true hereditary hemophilia.

Bullock and Liles,<sup>1</sup> in a survey of 600 hemophilic families, could find no evidence of the disease among the female subjects. Birch<sup>2</sup> in the United States reviewed seventy eight hemophilic families and could find no affected women among them. Others<sup>3</sup> have made similar investigations and have found no true female hemophiliacs. Bueuri<sup>4</sup> collected 197 cases of "female hemophiliacs" from the literature but he found the evidence equivocal and inconclusive for such a diagnosis in all of the cases. As Quiek<sup>5</sup> points out all of the cases of so called hemophilia in women, antedating the very recently reported ones, must be restudied in the light of newer diagnostic procedures and the diagnosis cannot rest on history and a prolonged coagulation time of the blood alone. According to Quiek<sup>5</sup> "It is extremely doubtful whether a true case of hemophilia has ever occurred in a woman."

In 1945 Madison and Quiek<sup>6</sup> described this disease entity as "hemophilia like disease in the female" and gave in account of a woman aged 30 who developed hemorrhage into the deep tissues of the body and evidence of hematoma. She had a prolonged coagulation time, but the coagulation time

of recalcified plasma was normal. She developed severe hemorrhage into the tongue and the pharynx which led to mechanical obstruction of respiration and subsequent death. Autopsy revealed nothing other than extensive hemorrhage throughout the body. These authors also reported the case of a woman, aged 33, in whom hemorrhagic manifestations appeared one year following childbirth. The hemorrhages were for the most part superficial, with epistaxis and one attack of hematuria. The prothrombin time was 88 per cent, and the coagulation time was one hour and fifty minutes. The family history was negative. Joules and MacFarlane<sup>7</sup> in 1938 described the similar type of bleeding in a 56-year-old woman. Following extraction of a tooth she developed hemorrhages into the subcutaneous tissue, hematuria, and bleeding into the tongue and joints. She had a prolonged coagulation time. When Russell's viper venom was added to the blood in the tube the coagulation time became almost normal. Other cases of female bleeders, such as those reported by Kubo,<sup>8</sup> Schultz,<sup>9</sup> and Chaigaff and West<sup>10</sup> resemble hemophilia more closely than they do any other hemorrhagic disease. It is obvious that these along with the two we have reported, belong to an entity that has a coagulation defect in the blood not unlike that of true hemophilia.

Whether or not an inhibitor substance is present in the blood of hemophilic persons accounting for the hypocoagulability has not been conclusively determined. Recently Tocantins<sup>11</sup> has presented evidence suggesting that the primary disturbance in hemophilia is associated with an excess of antithromboplastin. Others<sup>12</sup> hold that the theory of an anticoagulant as the cause of hemophilia is untenable. The presence of such an anticoagulant has been suggested as an explanation for the hypocoagulability of other hemorrhagic diseases. Such an anticoagulant had never been observed in human blood, however, until Lozner, Jolliffe, and Taylor<sup>13</sup> reported a patient with a prolonged coagulation time which they proved to be associated with an anticoagulant in the blood. Therefore, these authors suggested that such bleeding problems be investigated and a search made for the presence of an anticoagulant in the plasma. Fantl and Nance<sup>14</sup> also have reported a woman who acquired a hemorrhagic diathesis which they proved to be due to a specific antithromboplastic factor.

Following the technique described by Lozner, Jolliffe and Taylor,<sup>13</sup> we have attempted to exclude the possibility of an anticoagulant in our second patient. For this experiment blood was obtained from a normal donor and from our patient. The plasma was prepared from both blood specimens by adding sodium citrate to the whole blood so that the final citrate concentration was 0.25 per cent. The blood was then centrifuged, the plasma was removed and filtered through a Seitz filter. The normal blood had a clotting time of nine minutes. When 0.1 ml of filtered citrated plasma from our patient was added to 2 ml of normal whole blood, it continued to clot at the same rate of nine minutes. This seems to eliminate the possibility of the presence of an anticoagulant in the blood plasma as the basis for the retarded coagulation time.

The next experiment was made with blood obtained from the same patient a normal donor and a hemophilic patient. The same technique was followed in preparing the plasma. When 0.1 ml of normal citrated plasma was added to 2 ml of hemophilic blood there was an acceleration of the coagulation time from fifty seven minutes to twelve minutes. Under the same conditions 0.1 ml of normal citrated plasma was added to 2 ml of the patient's blood. There was a similar acceleration of the clotting mechanism and the coagulation time was reduced from 130 minutes to thirty minutes. We next added 0.1 ml of plasma from the hemophilic subject to 2 ml of blood obtained from the patient. The time required for the coagulation to occur was not altered remaining at fifty seven minutes. (These figures are summarized in Table I.) These facts seem to indicate that normal plasma contains a substance which is absent or deficient in the blood of the hemophilic person and of our patient. Whether the deficient factor is the same in both instances is not known.

Some of the more recent work by Patel and Taylor<sup>1, 2</sup> and then as associates<sup>3, 4</sup> has shown that normal cell free human plasma contains an active principle which when added in small amounts to hemophilic blood produces normal coagulation. This substance is closely allied with the globulin fraction of the plasma proteins and has been designated the 'globulin substance'. They were unable to obtain it in any significant quantity from hemophilic plasma. Howell<sup>5</sup> has confirmed these observations but prefers to call the material plasma thromboplastin. There seems to be fairly conclusive evidence that there is a deficiency in the plasma of hemophilic patients which accounts for the prolongation of the clotting time. More recent investigation suggests that this globulin fraction acts as the progenitor of an enzyme system which plays an as yet unknown role in the process of blood coagulation. In hemophilic plasma the enzyme system may be present in reduced amounts. Along this line Quick<sup>6</sup> has recently presented evidence that the platelets are normal in hemophilia liberating their enzyme in a normal manner. He proposes that this platelet enzyme acts directly on the precursor of thromboplastin which he designates thromboplastinogen. Hemophilic patients thus lack this precursor thromboplastinogen which is normally present in the plasma.

We were therefore interested in seeing whether any disturbance in the plasma protein fractions could be demonstrated. Employing the technique of Tiselius\* we determined the various plasma proteins. These are recorded in Table IV. In our first patient there was a low value for the total protein and for the albumin which could be accounted for by blood loss. The globulin fractions and fibrinogen were within the range of normal although the alpha globulin was at the lowest value of normal. In our second patient the total protein was normal the albumin was normal but the alpha globulin was abnormally low. The beta and gamma globulin and the fibrinogen fractions

\* Longworth's<sup>7</sup> modification of the Tiselius electrophoretic method was used to estimate the albumin,  $\alpha$  globulin,  $\beta$  globulin and  $\gamma$  globulin and fibrinogen in the plasma. Total protein values were determined by Pregl's modification of the micro Kjeldahl method for estimating total and nonprotein nitrogen.

TABLE IV TISELIUS PROTEIN FRACTIONATIONS (GM/ML)

NORMAL RANGE	TOTAL	ALBUMIN	$\alpha$ GLOBULIN	$\beta$ GLOBULIN	$\gamma$ GLOBULIN	FIBRINOGEN
High	7.82	5.11	0.66	1.07	0.91	0.48
Low	5.94	3.72	0.39	0.65	0.60	0.16
Case 1	5.30	2.78	0.39*	0.80	0.91	0.42
Case 2	6.40	4.21	0.31*	0.88	0.72	0.28

\*Definitely low values

were normal. Both of these protein patterns can we believe, be considered abnormal. The alpha-globulin fraction has been reported low in hypothyroidism, and hypopituitarism, which neither of our patients had. The significance of this discrepancy in the alpha-globulin is not known.

## SUMMARY

1. The clinical picture in two cases of hemophilia-like disease in women was similar to that found in true hemophilia and the outstanding characteristic was the prolonged coagulation time of the blood.

2. The coagulation time of recalcified plasma, which Quick<sup>27</sup> believes pathognomonic for hemophilia, was positive in both patients.

3. When normal citrated plasma was added to blood from one of our patients the coagulation was markedly accelerated. This is similar to the response of hemophilic blood to normal plasma.

4. Tiselius protein fractionation revealed definite abnormality in the alpha-globulins in one patient and suggestive but not conclusive evidence of abnormality in the second patient. We believe that these changes are significant.

5. The possibility that some acquired change in the plasma protein pattern might be the basis for the coagulation defect in our patients is suggested. The defect seems similar to that found in hemophilia.

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## THE LYMPHOCYTE

STUDIES ON ITS RELATIONSHIP TO IMMUNOLOGIC PROCESSES IN THE CAT

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THE part played by the lymphocyte and lymphoid tissue in the normal physiology of the body and in the response of the latter to injurious stimuli and disease states has been the subject of many speculative theories, but a surprising paucity of well-established information exists in regard to the functions of this widespread tissue. It is quite apparent that the lymphocyte must be of considerable significance in the general body economy if one but considers the rate of utilization and concomitant production of lymphocytes in the normal animal. It is reasonably well established that each lymphocyte must yield its metabolically important contents within a few hours after entering the blood stream<sup>1, 2, 3</sup>. Recent investigations have directly implicated the lymphocyte and lymphoid tissue in important immunologic functions—a relationship which previously had been postulated many times but never adequately substantiated experimentally. Because of the great significance of such a concept in regard to clearer understanding of the body's defense processes, thorough evaluation of the existing knowledge is essential.

Many and diverse sites of antibody formation have been implicated by different investigators. Reticulo-endothelial tissue has attracted particular attention in this regard. Thus Hartley<sup>4</sup> reported evidence of antibody formation occurring in the histiocytic cellular reaction about the intradermal injection of an aluminum hydroxide gel suspension of vaccinia virus. Experiments by Topley<sup>5</sup> in 1930 seemed to indicate that the "histiocytes of the spleen themselves form antibodies or some intermediate product." Similarly De Gara and Angevine<sup>6</sup> found antibody in tissues rich in reticulo endothelial cells before any antibody appeared in the blood. The inhibition of antibody response by any procedure which severely injures the cells of the reticulo-endothelial system<sup>5, 10</sup> is further indirect evidence of the role of the latter in the formation of antibodies. Tissue culture experiments likewise support the contention that reticulum cells such as those found in bone marrow lymph nodes, and spleen are capable of producing antibodies. In fact, Meyer and Loewenthal,<sup>11</sup> employing the rabbit, reported antibody formation from cultures of omental milk spots, a tissue which contains only cells of the reticulo-endothelial type. The plasma cell has also been felt to take part in immunologic processes<sup>12, 13</sup>. However, there is disagreement as to the primary importance of the plasma cell in antibody formation<sup>14</sup>.

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In spite of the diversity of opinion as to the relative importance of various tissues in the antibody mechanism, recent findings revive the concept that lymphoid tissue and its product, the lymphocyte, are fundamental in the process. In 1935 McMaster and Hindack<sup>1</sup> <sup>16</sup> first reported evidence of agglutinin formation in the local lymph nodes during the area of intradermal injection of antigen into mice before detectable antibody appeared in the blood. Similar results later were obtained in rabbits. Since then numerous workers<sup>17</sup> <sup>18</sup> <sup>19</sup> have concluded that lymphocytes contain gamma globulin and hence are potential carriers of antibody protein. Ehrlich and Harris<sup>14</sup> and Harris Gimm, Mertens and Ehrlich<sup>6</sup> conducted experiments to show antibody appeared in the efferent lymph of the popliteal lymph nodes of rabbits after local intradermal injection before the rise in serum antibody titer. They also presented evidence<sup>6</sup> <sup>1</sup> to show that the antibody was actually contained within the lymphocytes and they concluded that antibody was either manufactured in the cell or was concentrated therein and transported to the blood by the lymphocyte.

These conclusions are in sharp contrast to those reached by Hektoen in 1911. This investigator followed the rise in antibody titer in dogs after amputation of the leg into which antigen had been injected subcutaneously three to forty eight hours previously and found that the rise was comparable to that in normal controls. These findings would indicate that other sites of antibody formation can fully compensate for any antibody which may have been produced in local areas during the site of antigen injection, even though the latter have been ablated. They also indicate the rapidity with which antigen was spread throughout the body after the initial injection.

Dougherty Chase and White have presented substantiating evidence for the presence of antibody within lymphocytes.<sup>3</sup> They have also extended the concept of pituitary-adrenal cortical control of lymphoid tissue structure and function to embrace control of antibody release from tissue cells.<sup>3</sup> <sup>28</sup> An analysis of the evidence for the concept of adrenal cortical hormonal control of lymphoid structure and function has been the subject of a previous paper<sup>9</sup> and extensive résumé of related work would be unwarranted here. There is little doubt that increased availability of C11 oxygenated adrenal cortical steroids is associated with definite histologic degenerative changes in lymphoid tissue. However, there is marked disagreement concerning the influence of these hormones on the functional capacity of lymphoid tissue. In some animal species adrenal cortical hormone seems to influence the peripheral blood lymphocyte count.<sup>30</sup> <sup>34</sup> In other species proof of a specific influence is yet forthcoming.<sup>9</sup> <sup>3</sup> And in man there is at present conflicting evidence in regard to the existence of a pituitary-adrenal cortical mechanism for regulating the level of circulating blood lymphocytes. The hematologic picture in patients with Cushing's or Addison's disease<sup>3</sup> suggests a possible relationship between the relative numbers of lymphocytes and the activity of the adrenal cortex. In absolute numbers however, the alterations in lymphocytes are not as striking as the differences in the polymorphonuclear leucocytes and it



is difficult to evaluate the nonspecific secondary effects associated with the general metabolic disturbances in such conditions. In the hands of some investigators<sup>36</sup> a significant lymphopenia has been found to occur in patients with normal adrenal function after the administration of adrenocorticotrophic hormone. Others<sup>37</sup> have not observed any hematologic alterations specifically referable to a direct action of adrenal hormones on lymphoid tissue. No increment of serum gamma globulin or augmented antibody response has been found to follow the administration of adrenocorticotrophic hormone in human beings, as reported by Dougherty and White<sup>38</sup> in animals.

The influence of adrenal cortical hormones on the rate of production of lymphocytes by lymphoid tissue is also controversial. Some workers<sup>38-39</sup> have reported a significant reduction in the number of thoracic duct lymphocytes produced per unit of time after administration of pituitary adrenotropic hormone. Data collected in this laboratory failed to show any significant effect of adrenal cortical hormone on the rate of production of lymphocytes in normal and adrenalectomized cats.<sup>29</sup>

In spite of the lack of conclusive data concerning the influence of adrenal cortical hormone on the productive capacity of lymphoid tissue, considerable evidence exists to suggest that alterations in availability of adrenal cortical hormone are of importance in the immunologic response of animals.<sup>20, 28</sup> Dougherty and co-workers have reported an appreciable augmentation of serum antibody titer in mice by injections of adrenal cortical hormone or by exposure of the animals to various noxious stimuli designed to promote increased hormonal secretion by the adrenal cortex, such as roentgen radiation.<sup>40, 41</sup>

Other investigators have not all been able to demonstrate the potentiating influence of adrenal cortical hormones on antibody formation. Experiments performed in this laboratory in connection with the effects of x-ray on immunologic processes in rabbits<sup>42</sup> failed to show any alteration in the level of serum antibody by exposure to either small or large doses of x-ray, in spite of marked destruction of lymphoid tissue. Eisen and associates,<sup>43, 44</sup> using quantitative serologic methods, could detect no alteration in serum globulin or antibody as a result of alterations in availability of adrenal cortical hormone in mice. Houghton and co-workers<sup>45</sup> could not find any impairment of the response of cats to antigenic stimuli by adrenalectomy, and Murphy and Sturm<sup>46</sup> obtained data which suggested an actual augmentation of the immune response following the procedure. It is therefore apparent that opinion differs in regard to the hormonal control of antibody release from lymphoid tissue.

#### RATIONALE OF EXPERIMENTAL APPROACH

The purpose of the present work can be summarized under three headings. First, an attempt has been made to repeat under different experimental conditions the work of previous investigators insofar as the presence of antibody protein in lymphocytes is concerned. Second, we have studied the influence

of sudden extensive destruction of lymphoid tissue during the early phases of antibody production upon the antibody content of the lymph and blood serum. The tool used for this destruction was massive total body roentgen irradiation. Third in extension of this latter phase of the experiment was the study of the effect of large doses of adrenal cortical extract on the antibody content of thoracic duct lymph. Previous experiments had failed to show any significant effect of the administration of adrenal cortical hormone on the numbers of cells in thoracic duct lymph in cats. Consequently experiments on the effect of hormone administration have not represented one of the main features of this investigation. However a limited amount of data was collected pertaining to the influence of large doses of adrenal cortical extract on antibody content of lymph and this has been included in this report.

### *Experimental Procedure of Immunization, Cannulation and Lymph Collection*

The experimental animal used throughout was the cat. A standard antigen of typhoid vaccine (1 000 million killed organisms per cubic centimeter) prepared by the New York State Department of Health Laboratories was used for immunologic purposes. Blood samples were withdrawn prior to immunization. Each cat was given 0.2 cc vaccine subcutaneously followed by a similar amount intraperitoneally the next day. Four days after the first injection the cat underwent thoracic duct cannulation. This time interval was chosen because it was advisable that the studies should be undertaken during the early stages of antibody formation. Harris and co-workers<sup>2</sup> found that in their hands the maximal differences between cell antibody titer and that of the lymph and serum were detected around four to five days after inoculation. All cats used showed a definite serum antibody titer at this time.

Cannulation was performed under pentobarbital sodium anesthesia using the same operative technique as previously described. Lymph was allowed to flow freely from the small glass (silicone coated) cannula into a graduated 15 cc centrifuge tube containing a small amount of 1 per cent sodium heparin in saline. Care was taken to prevent contamination of the lymph by blood or serum and any specimen so contaminated was discarded. Hydration of the animals was maintained by subcutaneous injections of isotonic saline in amounts of approximately double that lost as lymph. The lymph collected was then subjected to the studies to be described.

### *Experimental Procedure to Demonstrate the Presence or Absence of Antibody in Lymphocytes*

I.—After immunization, each of the six animals in Group I was subjected to thoracic duct cannulation. Lymph was collected as a single volume for the entire period of lymph drainage (five to seven hours). The lymph was then centrifuged until all the cells had been removed from the supernate. A specimen of this cell free lymph was stored in the frozen state for titration.

The packed lymphocytes were resuspended in a volume of heparinized saline approximately equal to the original volume of lymph and recentrifuged. This washing process was carried out three times in large volumes of the heparinized saline care being taken with each washing to break up gently the clumps of lymphocytes so that they would be washed free of any lymph fluid. The final fourth washing was performed with approximately 14 cc of saline in 15 cc centrifuge tubes. A specimen of this last washing fluid

after centrifugation was preserved for titration. The total volume of fluid was then reduced to 125 cc, and the packed, washed cells in the bottom of the tube were resuspended carefully in this small volume. Cell counts and smears were made on this suspension after mixing. It was not possible in every instance to separate completely all clumps of lymphocytes by agitation before counting. In such cases, the counts must be regarded as only rough approximations. Nevertheless, it can be observed from Table I that all suspensions were extremely cellular, the lowest total count per millimeter<sup>3</sup> being 47,000 cells and the highest 178,500 cells. Smears made from the suspensions showed good preservation of lymphocyte morphology in all cases and revealed that lymphocytes comprised almost the total number of cells in each suspension. After counts were obtained, the cell suspension was again centrifuged, and the supernatant saline removed. One cubic centimeter of distilled water was added to the packed washed lymphocytes, and partial lysis of the lymphocytes resulted.

Before titrations were carried out, both the specimen of cell free lymph and the suspension of lymphocytes in distilled water were subjected to alternate rapid freezing and thawing six times, employing a dry ice acetone solution as the freezing media. Both cell free lymph and the lymphocyte suspension were similarly treated in order to eliminate any possible effect of the procedure or antibody protein as a source of difference between the titers of the two. After lysis in this manner, the remaining emulsion was subjected to centrifugation to remove the particulate cell stroma which would render performance of agglutination tests difficult. Titrations were then carried out (Wadsworth<sup>4</sup>) on the cell extract, the cell free lymph, the final washing fluid, and on serum collected before immunization, at the onset of cannulation and at the end of the experiment. Standard "H" (flagellar) and "O" (somatic) antigens prepared by the New York State Department of Health Laboratories were used. The first dilution of cell extract, lymph, or serum was 1 to 10 for this series of titrations. Subsequent dilutions progressed by double in each tube. The tubes were incubated at 37° C for twelve to eighteen hours. The wide variation of serum antibody titer from animal to animal is indicative of the individual differences in the degree of immunologic response.

**Results** As shown in Table I, in none of the six cats included in Group I was it possible to detect antibody in extracts of rich suspensions of washed lymphocytes. Each animal, however, had a demonstrable antibody titer in both the blood serum and lymph fluid at the time of cannulation. In two instances (Cats 694 and 712) the blood serum was found to contain agglutinating substances for typhoid "O" antigen prior to experimental immunization. These two animals probably should not, therefore, be regarded as comparable to the larger group. Nevertheless, in no instance no matter what the titer of antibody in the blood serum or lymph, was any antibody found within the extract of lymphocytes.

It will be noted that in almost every instance the titer of the cell-free lymph fluid was slightly lower than that of the blood serum. The absence of any titer in the final washing fluid demonstrates that any detectable amount of antibody had been washed free of the lymphocytes by the procedure. It will also be observed that in three instances there was a significant rise in serum antibody concentration during the course of thoracic duct drainage. The possible importance of this rise will be discussed later.

The completely negative titers in the extracts of these highly cellular lymphocyte suspensions indicate the lack of titrable antibody within the lymphocytes as studied under the conditions of the experiment.

TABLE I. COMPARATIVE TITRATIONS OF SERUM CELL FREE LAMIN AND EXTRACTS OF PIGEON, WASHED LYMPHOCYTES IN A STUDY OF THE ANTIBODY CONTENT OF LYMPHOCYTES

GROUP I CAT	SERUM ANTIBODY TITER										NUMBER OF LYMPHOCYTES AFTER WASHING AND CONC (PER MM <sup>3</sup> )	TITER OF LYMPHOCYTE EXTRACTS		TITER OF CELL FREE FRACTION		TITER OF LAST WASHING FLUID	
	BEFORE IMMUNIZA- TION		AT TIME OF CANNULATION		AT END OF EXPERIMENT												
	H	O	H	O	H	O											
700	0	0	0	1/80	1/20	1/80	78,500†	0	0	0	1/10	0	0	0	0	0	
702	0	0	1/160	1/2,500	1/640	1/2,500	178,500†	0	0	0	1/20	1/320	0	0	0	0	
707	0	0	1/40	1/640	1/160	1/640	110,000	0	0	0	1/40	1/80	0	0	0	0	
095	0	0	1/80	1/160	1/80	1/320	47,000	0	0	0	1/20	1/80	0	0	0	0	
094	0	1/80	1/640	1/320	1/640	1/320	162,500	0	0	0	1/160	1/320	0	0	0	0	
712	0	1/100	1/2,500	1/640	1/2,500	-----	147,500	0	0	0	1/1,80	1/160	0	0	0	0	

The lymphocytes in each specimen represent all cells in the lymph collected over a four to six hour period washed four times and resuspended in 1 c.c. of fluid.

†Cells in clumps on counting chamber so figure is only an estimate of the true count.

TABLE II COMPARATIVE TITRATIONS OF SERUM, CELL FREE LYMPH, AND CELL RICH LYMPH SPECIMEN REPRESENTS A

GROUP II	SERUM ANTIBODY TITER				LYMPH SPECIMEN FIRST TWO HOUR PERIOD BEFORE LYSIS				1,700 r Whole Body X ray	LYMPH SPECIMEN FIRST TWO HOUR PERIOD AFTER LYSIS			
					ANTIBODY TITER					ANTIBODY TITER			
	AT TIME OF CANNULATION		AT END OF EXPERIMENT		CELL FREE LYMPH		CELL RICH LYMPH			CELL FREE LYMPH		CELL RICH LYMPH	
	H	O	H	O	H	O	H	O		H	O	H	O
CAT													
549	1/40	0	1/40	0	1/80	0	1/40	0		1/80	0	1/40	0
608	1/1,280	1/1,280	1/2,560	1/320	1/160	1/320	1/160	1/80		1/320	1/80	1/160	1/160
582	1/80	1/160	1/160	1/160	0	1/20	0	1/20		1/20	1/40	0	1/20
651	0	1/320	1/20	1/320	0	1/40	0	1/40		0	1/40	0	1/80
641	1/20	1/80	1/40	1/1,280	0	1/80	0	1/40		0	1/40	0	1/40
657	1/1,280	1/160	1/5,120	1/320	1/320	1/20	1/80	-----		1/160	1/40	1/160	1/40
643	1/320	1/320	1/320	1/2,560	1/20	1/80	1/20	1/80		1/40	1/80	1/80	1/80
676	1/20	1/80	1/160	1/160	1/20	1/40	1/40	1/40		1/40	1/20	1/40	1/40

\*Figure represents lymphocytes per cubic millimeter in 1 cc of cell-rich lymph

II—In the following experimental approach, the repeated washing and handling of the lymphocytes was eliminated, excluding this as a possible source of loss of lymphocytic antibody protein during the procedures. The lymph from each cat was collected in two hourly volumes, and the amount of each specimen recorded. After thorough mixing of the lymph for five minutes, duplicate cell counts were made and the specimen then was centrifuged. Approximately 1 cc samples of the supernatant, cell free lymph were withdrawn. The remaining volume of the supernate was reduced to about 1 cc by pipetting off the excess cell free lymph. The packed cells in the bottom of the centrifuge tube were resuspended and thoroughly mixed in the remaining 1 cc of lymph fluid for five minutes. White cell counts were made from this specimen to determine the cell concentration and a smear was made to study the cellular morphology. In each case lymph was collected and handled in this fashion every two hours for the duration of the experiment. Blood specimens for titration were obtained just before cannulation and at the completion of the experiment.

As in the previous experiment, the lymph in each specimen collected for titration was alternately frozen and thawed six times. The cell free and cell rich specimens were each subjected to this procedure in order to eliminate alteration of antibody by the lysing process as a possible source of any significant difference in antibody titer between the two. In most instances the cell rich lymph contained a gelatinous residue after lysis of the cells and this was removed by a stirring rod. Titrations against standard "H" and "O" antigens were then carried out for each specimen and were read the next day (twelve to eighteen hours later).

Every cat used showed a low but detectable serum antibody titer at the time of cannulation. This titer varied widely from animal to animal, thus indicating that each animal was individual in the rapidity with which its immunologic mechanism responded to the antigen. However all must have been in the initial phases of antibody production since the time elapsed from the onset of immunization was so short. No attempt was made to compare the antibody responses of different cats each animal representing a distinct experiment.

## SUSPENSIONS BEFORE AND AFTER EXPOSURE TO 1500 r WHOLE BODY X RAY, FRESH LYMPH TWO HOUR COLLECTION

LYMPH SPECIMEN SECOND TWO HOUR PERIOD AFTER X RAY		LYMPH SPECIMEN THIRD TWO HOUR PERIOD AFTER X RAY		LYMPH SPECIMEN FOURTH TWO HOUR PERIOD AFTER X RAY	
ANTIBODY TITER		ANTIBODY TITER		ANTIBODY TITER	
CELL FREE LYMPH	CELL RICH LYMPH	CELL FREE LYMPH	CELL RICH LYMPH	CELL FREE LYMPH	CELL RICH LYMPH
H O	H O	H O	H O	H O	H O
1/160 0	1/80 0 13,300	1/320 1/20	1/3 1/160 -00	1/320 0	1/320 0 15,700
1/3 0 1/160	1/320 1/320 2,000	1/320 1/160	1/16 1/160 40,200	1/640 1/160	1/640 1/160 8,900
1/40 1/40	1/20 1/40 77,000	1/40 1/10	1/40 1/40 -2,000	1/40 1/80	1/40 1/40 15,400*
0 1/40	0 1/40 21,000	0 1/40	0 1/40 1,500	0 1/40	0 1/40 11,700*
0 1/20	0 1/20 24,000*	0 1/40	0 1/20 -3,300	1/20 1/40	0 1/40 26,500
1/320 1/40	1/320 1/40 56,450	1/320 1/10	1/16 1/40 1,000	1/160 1/40	1/320 1/40 17,100
1/40 1/40	1/80 1/40 45,000*	1/40 1/40	1/40 1/40 7,150*	1/80 1/40	1/40 1/80 4,050
1/40 1/80	1/40 1/40 1,000	0 1/80	1/20 1/80 5,700	1/40 1/40	1/40 1/40 6,900

**Results** The animals used for this phase of the experiment are included in Groups II and III. The data collected are presented in Tables II and III. Comparative antibody titrations were made between the cell free and lysed cell rich fractions of fifty seven separate specimens of lymph. Since titrations were made against both typhoid 'H' and typhoid 'O' antigens, this represented a total of 114 comparisons between the antibody content of cell free lymph and that of the lysed cell rich fraction of the same specimen. Of this total number of comparative titrations there was no significant difference between the antibody concentration of cell rich and cell free lymph specimens except in five instances. In three of these the antibody titer was higher in the cell free lymph than in the lymph which had been rich in cells before lysing. It is apparent, therefore that in only two instances did the lysed cell rich specimen contain significantly more titrable antibody than the cell free lymph plasma. In view of the small percentage of comparisons indicating a higher concentration of antibody in the cell extract and the roughly equal percentage of comparisons yielding the opposite results it is quite apparent that no valid evidence of the presence of antibodies within the thoracic duct lymphocytes was obtained in these experiments.

It is interesting to note the extremely high lymphocyte concentration in some of the specimens examined. Thus one special collection (not included in Table II) contained 550,000 cells per millimeter<sup>3</sup> of lymph. The antibody titer of this sample after lysing of the cells was "H" 1/160 and "O" 1/320. The titer of the cell free portion of the same collection was "H" 1/80 and "O" 1/640. It is evident that no significant difference in antibody concentration existed between the two samples of lymph. Other cell rich specimens were obtained one with a count of 311,000 lymphocytes per millimeter<sup>3</sup> and

TABLE III COMPARATIVE TITRATIONS OF SERUM, CELL FREE LYMPH, AND CELL RICH LYMPH EXTRACT,

GROUP III	SERUM ANTIBODY TITER				LYMPH SPECIMEN FIRST 2 HOUR PERIOD BEFORE HORMONE				25 cc Adrenal Cortical Hormone	LYMPH SPECIMEN FIRST 2 HOUR PERIOD AFTER HORMONE			
					ANTIBODY TITER					ANTIBODY TITER			
	AT TIME OF CANNULATION		AT END OF EXPERIMENT		CELL FREE LYMPH		CELL RICH LYMPH			CELL FREE LYMPH		CELL RICH LYMPH	
					H	O	H	O		H	O	H	O
CAT	H	O	H	O	H	O	H	O	H	O	H	O	
650	1/20	1/40	1/40	1/20	0	1/40	0	1/80		0	1/20	0	1/20
							15,400*					9,400*	
661	1/40	1/80			0	1/40	0	1/40		0	1/40	0	1/40
							95,500*					41,500*	
595	1/80	1/640	1/640	1/1,280	1/80	1/80	1/40	1/80		1/40	1/80	1/40	1/160
							140,000*				232,500*		
584	1/40	1/640	1/160	1/1,280	0	1/40	0	1/40		0	1/40	0	1/40
							206,000*					136,500*	

\*Figures represent lymphocytes per cubic millimeter in 1 cc cell-rich lymph

six with concentrations from 100,000 to 200,000 cells per millimeter<sup>3</sup>, and seventeen with counts between 40,000 and 100,000 per millimeter<sup>3</sup>. In no instance was the antibody concentration of any of these cellular lymph suspensions higher than that of the cell-free supernate from the same lymph collection. These data are included in Tables II and III.

In addition to the twelve animals included in Groups II and III there were three animals who were subjected to the same procedure but were not included for analysis. The results obtained from one cat were excluded because the presence of a specific serum antibody titer before immunization did not conform with the negative preimmunization titer for the other animals. Another experiment was discarded because all the titrations with typhoid "O" antigen were overgrown with *Bacillus pyocyaneus*. A third animal failed to develop a detectable antibody in the lymph. It is noteworthy in regard to this animal, however, that the serum antibody titer was positive at the onset of cannulation ("O" 1/20) and rose significantly during the period of lymph drainage ("O" 1/80 at end of experiment). In spite of this, no antibody could be detected in any of the lymph specimens throughout the day. Two other animals were studied for an insufficient period of time to be included in the group for analysis. One of these had been hyperimmunized with typhoid vaccine prior to cannulation and demonstrated a high antibody titer of equal degree in both serum and lymph.

None of these animals yielded any results which conflicted with those included in Tables II and III. Thus, in no instance were the data indicative, or even suggestive, of the presence of more antibody in the lymph containing extracts of lymphocytes than in the cell-free lymph.

#### *The Effect of Massive Whole Body X-ray on the Antibody Content of Lymph*

The animals included in Group II each were exposed to 1,500 whole body x ray two hours after cannulation, a preliminary lymph sample for base line determinations having been collected as described. The irradiation was delivered at 250 KVP at 15 Ma through aluminum parabolic plus 1/2 mm copper filters, the output being 28 r per minute and the

SUSPENSIONS BEFORE AND AFTER ADMINISTRATION OF 25 CC AQUEOUS ADRENAL CORTICAL SUBCUTANEOUSLY

LYMPH SPECIMEN SECOND HOUR PERIOD AFTER HORMONE		LYMPH SPECIMEN THIRD HOUR PERIOD AFTER HORMONE		LYMPH SPECIMEN FOURTH HOUR PERIOD AFTER HORMONE	
ANTIBODY TITER		ANTIBODY TITER		ANTIBODY TITER	
CELL-FREE LYMPH	CELL-RICH LYMPH	CELL-FREE LYMPH	CELL-RICH LYMPH	CELL-FREE LYMPH	CELL-RICH LYMPH
H O	H O	H O	H O	H O	H O
0 1/160	0 1/20 17,400	0 0	0 0 19 850	0 0	0 1/20 17,200
0 1/40	0 1/20 51,500	Animal expired		Experiment terminated	
1/80 1/80	1/40 1/40 100,000	1/80 1/80	1/80 1/80 42 800		
0 1/40	0 1/40 152 000	0 1/40	0 1/40 49 000	0 1/160	0 1/320 67 500

target distance twenty two inches to the center of the cat. This amount of x ray is much greater than the lethal range for cats and was chosen so that there would be no doubt that a maximal destruction of lymphoid tissue had been accomplished.

Lymph specimens were collected every two hours for eight hours after x ray and handled as described previously. At the end of this time blood was collected for antibody titration and the animal sacrificed. This amount of x ray causes a fairly uniform and progressive fall in thoracic duct lymphocytes over the eight hour period, so that the specimens collected toward the end of the experiment usually had a low lymphocyte content.

**Results**—The massive whole body roentgen radiation to which these animals were exposed was given with the intention of destroying large numbers of lymphoid cells over a short period of time. If lymphocyte precursors susceptible to damage by roentgen radiation contain significant antibody protein sudden extensive destruction of large numbers of these cells should result in a sharp rise in the antibody concentration of the lymph fluid. Since thoracic duct lymph normally contains relatively large numbers of cells, almost all of which are of the lymphocytic series such a phenomenon should be more easily demonstrable in lymph than in the blood.

In every case the lymphocyte content of the lymph fell progressively after exposure to x ray. This fall is a result of the destructive action of x ray on lymphoid tissue with impairment of lymphocyte production. An analysis of the effect of x ray on the rate of lymphocyte production has been the subject of another report<sup>48</sup> from this laboratory and will not be discussed here. Suffice it to say that the marked impairment of lymphocyte production is a reflection of degenerative changes brought about in lymph nodes by roentgen radiation.

In spite of the widespread destruction of lymphoid cells no appreciable alteration in antibody concentration of the lymph was detected. Thus, two hours after exposure to x ray, the antibody concentrations of both cell free and cell rich lymph specimens were approximately the same as those of cell free and cell rich lymph before x ray for every animal except one (Cat 608). In this instance the "O" titer of the cell free lymph fell from 1/320 to 1/40 after x ray. Such a reduction in titer is just the opposite of the theoretical response one



would expect if the damaging influence of x-ray were causing a release of antibody protein from the lymphocytes into the surrounding lymph. Titrations at the end of four hours were also approximately the same as before x-ray in all animals except Cat 582, which showed a positive "O" titer of  $\frac{1}{40}$  in the cell-free lymph at this time, whereas before x-ray no titer was detected. As shown in Table II, three of the animals (Cats 608, 549, and 582) showed a tendency for the lymph antibody titer to rise toward the end of the eight-hour period following x-ray. All the other animals showed no appreciable alteration in lymph

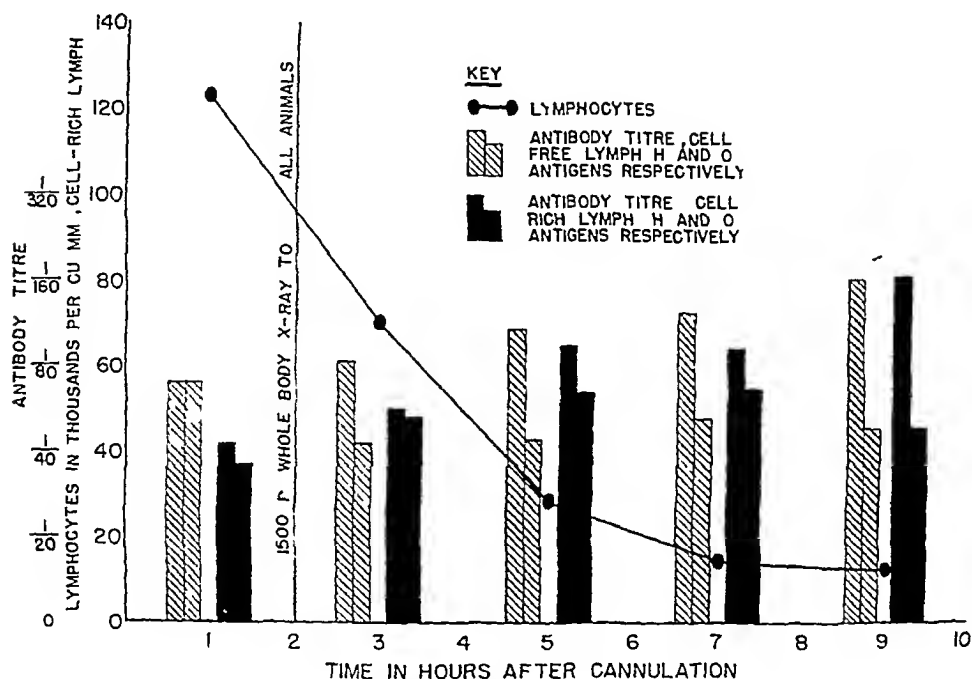


Fig 1—Graphic representation of the comparative antibody concentrations of cell-free and cell-rich lymph in nine cats the lymph specimens being collected every two hours. The values for lymphocytes represent the average number of cells in each two-hour lymph specimen per millimeter<sup>3</sup>.

antibody titer from the beginning to the end of the experiment. Fig 1 shows the reduction in the average numbers of lymphocytes in thoracic duct lymph after exposure to x-ray and the slight rise in average antibody titer of both cell-free and cell-rich lymph over the ten-hour period of thoracic duct lymph drainage. The significance of the slight rise in lymph antibody titer in three animals is not apparent. If it were the result of release of immune globulins from lymphocytes into the lymph fluid following x-ray, one would expect a more consistent and distinct elevation in the lymph of all, or at least most of the animals. Thus, in the experience of Dougherty and White<sup>11</sup> exposure of mice to x-ray was followed within two to three hours by a sharp and impressive rise in serum antibody titer. It is evident that in no single animal in this group was there a sharp rise in lymph antibody titer of any appreciable degree. In the three instances in which a significant rise in antibody titer after x-ray was

observed the greatest elevation was never more than two tubes (doubling the dilution in each tube). Conceivably the rise may have been due to hemorrhagic changes in the lymph nodes with diffusion of serum antibody protein into the lymph spaces. Or perhaps the protein concentration of the lymph became higher toward the end of the experiment in spite of water and electrolyte replacement. It is also possible since lymph fluid is derived from the extra vascular fluid that the rise in lymph titer like the rise in blood serum titer during the period of the experiment was not related to the lymphoid structures at all. Since these various possibilities were not controlled the mechanism of the slight antibody rise in the lymph of these three cats cannot be evaluated accurately. Lymphoid tissue destruction with release of antibody into the lymph is a possible explanation. However in view of the lack of consistent elevation of antibody titer in spite of extensive lymphoid tissue destruction in all cases the relative importance of such a mechanism in bringing about an increment in antibody concentration seems negligible.

The blood serum antibody titer rose significantly during the period of cannulation (ten hours) in four instances (Cats 641 657 643 and 676) or in exactly 50 per cent of the animals. The mechanism of this antibody increase in the serum in spite of the removal of the major portion of all lymph contents and the functional ablation of all lymphoid tissue by x-ray is of considerable speculative interest. This will be considered further in the discussion of the results. It will be noted that in one instance (Cat 608) there was a significant decrease in the serum antibody titer against typhoid "O" the explanation for which is not apparent.

No consistent correlation was found to exist between the height of the serum antibody titer and that of the lymph. Thus in several instances no antibody could be detected in the lymph in spite of a significant serum antibody titer. The concentration of serum antibody at the end of the experiment was higher than that of the lymph in all cases except one (Cat 549).

#### *Experimental Procedure to Demonstrate the Effect of Adrenal Cortical Hormone on the Antibody Content of Lymph*

The animals in Group III each were given 25 cc of aqueous adrenal cortical extract (Uppjohn) subcutaneously two hours after the onset of cannulation. No attempt was made to administer adrenal cortical extract per kilogram of body weight. However the amount given was much more than that previously reported as required for maximal effect on lymphoid tissue. Lymph was collected in two hourly volumes after the hormone injection and treated as in the case of those animals exposed to x-ray. Serum for titration was obtained at the beginning and end of cannulation.

**Results**—The failure of adrenal cortical hormones to influence the rate of production of thoracic duct lymphocytes in cats has been previously demonstrated.<sup>10</sup> As indicated in these previous experiments a fall is noted in the numbers of lymphocytes delivered via the thoracic duct per unit of time in normal untreated animals as a result of the procedure of cannulation itself. This initial fall is unrelated to adrenal cortical activity since it was shown to occur in animals previously adrenalectomized irrespective of whether or not

adrenal cortical hormone was administered. In the present series the same decline in the total lymphocyte content of lymph per unit of time after cannulation was noted but was not felt to be a result of adrenal cortical hormone administration.

It is apparent that in this limited series of experiments administration of adrenal cortical extract in large amounts had no effect upon the relative antibody concentration in cell free and cell-rich lymph. In no instance was a significant rise in the antibody titer of cell-free lymph found to occur within six hours after hormone injection. Three to six hours is the time interval after hormone administration for maximal effect on lymphoid tissue and antibody titer, as reported by previous investigators<sup>20</sup>. In one instance (Cat 584) a rise in lymph antibody titer at the end of the experiment was recorded. The mechanism of this rise, as in the case of the three irradiated animals who demonstrated the same phenomenon, is not completely clear. Here again it may have been the result of release of immune globulins from the cytoplasm of lymphocytes, but the possibility that other factors, previously discussed, may have entered into the picture cannot be excluded.

In two instances (Cats 584 and 650) a detectable serum antibody titer for typhoid "H" antigen was present although a negative lymph antibody titer was recorded. Also in two of the four animals of this group, a significant rise in serum antibody titer during the period of lymph drainage was recorded.

It should be re-emphasized that none of the comparisons of cell-free and cell-rich lymph in this group of animals revealed any higher antibody concentrations in the cell-rich fraction. It will be noted that there were relatively more specimens which were highly cellular for each animal in this group than in those exposed to X-ray.

#### DISCUSSION

It was the purpose of these experiments to study some of the features of antibody production by lymphoid tissue and its transport to the circulating blood by lymphocytes. It was recognized that the experimental approach differed from any employed in previous investigations of this nature. However, if the formation of antibody by lymphoid elements and the transport of antibody to the blood by lymphocytes represents a major factor in the total immunologic response of the animal, the technique used in these experiments should provide a valid means to study these phenomena. Thus, collection of all the lymphocytes and lymph draining from areas presumed to be participating in immune globulin production should yield detectable antibody in extracts of these lymphocytes, if they represent a major source of transport of antibody into the circulating blood. The thoracic duct represents the final main stream for the flow of lymph after the entrance of lymphatic tributaries from all regions of the body below the diaphragm. Since the antigen injections were intraperitoneal and into the subcutaneous area of the back it is apparent that at least part of the total lymph collected was draining from lymph nodes concerned in the uptake of antigen. The lymphocytes originating in this nodal tissue would presumably contain antibody

The absence of any detectable antibody in the extracts of concentrated suspensions of washed lymphocytes collected during a period of active antibody production can be interpreted only as indicative of the very low or negative titrable antibody content of lymphocytes as measured in these experiments. The negative results obtained by this technique were substantiated by the large number of comparisons of relative antibody content of cell free lymph fluid and lymph containing many lymphocytes which had been artificially lysed in order to release their contents into the lymph fluid.

It is not readily apparent why the experimental approach used should yield negative results in view of the positive findings of others in studies on the crude extracts of minced lymph nodes as well as on the lymphocytes collected from a single lymph node draining the site of antigen injection. The conflicting data may perhaps be partially explained by differences in experimental techniques. There are possible objections to these and other experimental methods which merit critical evaluation. For instance it might be argued that the method of cell lysis employed resulted in antibody remaining in some manner inalterably bound to cell stroma and incapable of appearing free in the fluid lymph. Such argument appears negated by the fact that similar technique has been employed with positive results in experiments by other investigators. It is also conceivable for example, that the amount of antibody protein within each lymphocyte may have been so small that detection would be difficult simply on the basis of the range of antibody concentration. This possibility was counteracted as adequately as possible by studying the antibody content of very large numbers of washed lymphocytes suspended in a relatively small volume of fluid. Under these conditions, detection of any antibody, even of low titer would be considered significant evidence of the presence of antibody within lymphocytes. On the basis of experiments by previous investigators in which high concentrations of antibody were found in extracts of washed, minced lymph node tissue, it was anticipated that the demonstration of significant amounts of antibody in cell rich suspensions would not be difficult. The complete absence of titrable antibody in any of these suspensions, however indicated that the presence of antibody within lymphocytes could not be detected by this technique. It was realized that even small amounts of antibody in each lymphocyte entering the blood would be significant because of the rapid turnover of these cells and the processes of body metabolism which conserve serum protein. Thus repeated contributions of antibody however small, if sufficient in number would ultimately result in substantial titers. It can be stated however, that under these circumstances detection of such small amounts of antibody in cells would be rendered very difficult by any approach.

There also is the possibility that the antibody protein within lymphocytes may exist in an incomplete form which has not developed specificity. In such an event detection by specific immunologic titration would be impossible. This contingency, which is not supported by any evidence, has not been circumnavigated by any method yet employed.

It may also be argued that antibody was not found in extracts of lymphocytes in these experiments since perhaps only a certain percentage of lymphocytes collected were produced in areas concerned with the initial uptake of antigen and subsequent antibody production. Following this line of reasoning, a small amount of antibody protein in some of the cells may have been obscured by the contents of other cells not transporting antibody. Such a possibility cannot be completely excluded. Nevertheless, as previously stated, it is likely that a large number of lymphoid areas participated in the uptake of antigen and that the products of these nodes entered the thoracic duct system. These lymphoid areas were presumed to be actively producing antibody at the time of cannulation. It would seem probable, if antibody formation by lymphatic tissue and transport to the blood by lymph represents a major factor in the elevation of serum antibody concentration that collection of all thoracic duct lymph and its contents for a several hour period during the stage of active antibody production would yield significant amounts of titrable antibody.

Species difference may conceivably have accounted for the negative results in cats as contrasted to the positive findings of previous investigators in mice and rabbits. The possibility that lymphoid tissue varies in its functions from one mammalian species to another can be clarified only by further investigation. The only conclusion reached from these experiments is that demonstration of antibody within lymphocytes of the cat was not possible using a technique basically similar to that employed by previous workers in different species.

The continued rise in serum antibody titer in eight of the animals during the course of thoracic duct drainage casts further doubt upon the necessity of lymphatic transport of antibody for the development of high serum antibody levels. The possibility exists that antibody protein formed within lymph nodes may have entered the blood stream directly through the vascular system of the nodes themselves. Diffusion of protein across capillary endothelial membranes from lymph is not thought to occur<sup>3</sup> and consequently actual intravascular production of antibody in lymphoid areas would seem necessary in this event. The concept under question, however, deals with antibody formed in lymph nodes and transported to the blood stream by the lymphocytes produced in the same area. The normal route of entrance of lymphocytes into the circulating blood is via the lymphatic system<sup>1, 2, 3</sup>. It is apparent that removal of practically all lymph before its entrance into the circulating blood eliminated lymphatic transport of antibody from any lymphoid tissue draining into the ductile system as a contributing factor in the subsequent rise of serum antibody concentration. However, it is appreciated that not all entrances of lymph into the circulating blood were blocked in these experiments. In performing the operation, small tributaries from the head and left foreleg were usually ligated. Small ducts from the right foreleg, head, and thorax may have had separate channels of entrance into the blood. However, not more than a small percentage of the total lymphoid tissue of the

body could have returned access to the blood through the normal channels and these few areas must have been relatively far removed from the site of antigen injections. If it is maintained that the increase in serum antibody titer after cannulation was due to the continued production of antibody by this small amount of lymphoid tissue whose products still had access to the blood by the lymphatic route then several observations are apparent which conflict with the concept of antibody formation by local lymphatic tissues. First it would be necessary to concede that antibody production had rapidly become a generalized process taking place in all lymphoid tissue rather than primarily in that tissue concerned with the uptake of antigen. Second the rate of antibody formation by this small amount of lymphoid tissue with an available lymphatic entrance into the blood would have to be very great and the concentration of antibody within the lymphocytes transporting it to the blood would necessarily be quite high. The possibility that the elevation in serum antibody could have depended upon the small amount of lymph still having access to the blood seems particularly remote in those experiments in which animals were subjected to heavy whole body irradiation. It seems reasonable to conclude little likelihood exists that the observed rise in serum antibody titers can be explained by such a mechanism.

There are other factors which may have been responsible for the continued rise in serum antibody in spite of removal of most of the lymph contents and cessation of antibody formation by lymphoid tissue. Thus, the rise may have been due to (1) continued immune globulin production by some tissue more radioresistant than lymphoid cells and not dependent upon lymphatic transport of its products to the circulating blood, (2) release of preformed antibody directly into the blood stream or (3) marked hyperglobulinemia as a result of hemoconcentration with a concomitant increase in antibody concentration per unit volume of serum without an actual rise in the total amount of circulating antibody. An attempt to combat this latter factor was made by prearterial administration of isotonic saline solution in amounts about double the volume of lymph lost. It is therefore improbable that hemoconcentration was of great importance in the elevation of serum antibody.

The relative importance of the first two mentioned mechanisms is of course, hypothetical. The data would indicate that release of significant amounts of antibody protein from cells of the lymphoid series does not occur even after massive x-ray radiation. Whether some other type of cell might release antibody protein into the serum when damaged by x-ray can not be judged from these experiments. That antibody production in rabbits can go on after lymphoid tissue has been severely damaged was indicated in previous experiments performed in this laboratory.<sup>1</sup> Thus exposure of the animals to large doses of x-ray five days after immunization failed to alter in any way the rate or degree of antibody appearance in the blood in spite of widespread damage to lymphoid tissue. In view of this evidence it is probable that continued production of antibody by some tissue not immediately

destroyed by x-ray offers the best explanation of the continued rise in the serum antibody in these animals

The exposure of these animals to massive doses of x-ray was based on the assumption that if lymphoid cells contain antibody protein the resulting cell damage would be reflected in alterations in the intracellular and extracellular antibody content. The extreme sensitivity of lymphoid elements to roentgen rays is well known. The amount delivered to these animals was far in excess of that required to cause widespread damage to lymphoid tissue and was used in order that the destruction of this tissue would be unquestionable. Of course, there is reason to believe that mature lymphocytes may be relatively undamaged by irradiation of this degree and that the chief site of damage is the progenitor cells. If the latter contain antibody in any appreciable amount, the extensive cell damage wrought by the x-rays would have released antibody into the surrounding fluid, provided that such a phenomenon can occur after cell destruction. If lymphocytic dissolution, as caused by roentgen irradiation is capable of causing such a rise in serum antibody titer, the phenomenon should, then, be all the more pronounced in lymph. The results, however, fail to show any sharp or distinct alteration in lymph antibody titer after x-ray exposure. It is therefore, unlikely that an increase in serum antibody could be attributed to release of antibody from lymphoid cells. That marked lymphoid tissue destruction had been brought about is evident from the rapid decline in lymphocyte production over the eight-hour period of thoracic duct clamping. The absence of any appreciable augmentation of antibody titer of the cell-free lymph was interpreted as supportive evidence for a lack of titratable antibody within lymphocytes.

The theoretical influence of excessive amounts of adrenal cortical hormones on lymphocytes, according to the concept of pituitary-adrenal cortical control of lymphoid structure and function, would be one of dissolution and degeneration. This lymphocytic dissolution has been stated to release gamma globulin from the cell cytoplasm into the serum, and in the case of the immunized animal, the result is said to be an elevation of the serum antibody titer. The amount of data collected in these experiments in regard to this influence of adrenal cortical hormones on antibody titer is relatively small. However, there was no evidence of any change in antibody titer attributable to hormone injection. The failure of both massive irradiation and the administration of adrenal cortical hormone to influence the lymph antibody titer in cats suggests that more work is necessary in elucidating the role of lymphoid tissue in the immunologic mechanism of different species.

#### SUMMARY AND CONCLUSIONS

1 A review of the existing knowledge of the relationship of lymphoid tissue and the lymphocyte to immunologic processes is presented. Brief analysis is made of the theory of pituitary-adrenal cortical control of lymphoid tissue structure and function.

2 An experimental approach is presented for the study of the antibody content of lymphocytes collected from the thoracic duct lymph of rats. Using the technique described, typhoid vaccine as an antigen, and the cat as the experimental animal, no antibody could be detected within extracts of washed lymphocytes. Comparative titrations of the relative antibody content of lymph fluid free of cells and lymph containing large numbers of lymphocytes which were artificially lysed in order to release their protein content into the surrounding lymph fluid also failed to indicate the presence of any antibody within the lymphocytes.

3 A group of cats was exposed to 1500 r whole body roentgen radiation during the period of thoracic duct drainage. This amount of x-ray is in excess of the dosage required for maximal lymphoid tissue destruction. Lymphocyte production diminished markedly after this procedure as evidenced by the fall in output of thoracic duct lymphocytes. Exposure of the animals to x-ray did not significantly alter the antibody content of the cell free lymph fluid. This is interpreted as indicative of the slight or negligible amount of antibody released from lymphoid tissue by x-ray damage of cells.

4 It was observed that the antibody concentration in the blood serum rose significantly during the period of thoracic duct drainage (six to eight hours) in 50 per cent of the animals. This was true in those animals exposed to x-ray as well as the others. This continued rise in serum antibody titer in spite of lymph drainage, particularly in those animals exposed to x-ray two hours after operation suggests either continued antibody formation by some more radioresistant tissue than lymphoid or release of preformed antibody from cells or tissue spaces directly into the blood without the necessity of lymphocytic transport of antibody.

5 Administration of large doses of adrenal cortical hormones (Upjohn) to a small group of cats failed to cause any significant alteration in the antibody content of cell free lymph fluid.

6 A discussion of the results is presented emphasizing the possible criticisms of the technique used. It is concluded that, under the experimental conditions of the experiments, no evidence for lymphocytic transport of antibody to the blood was found.

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# A STUDY OF CHOLINESTERASE ACTIVITY IN THE BLOOD OF PATIENTS WITH HEMATOLOGIC DISEASE

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NEW YORK, N Y

IT HAS been shown<sup>1, 2</sup> that red blood cell cholinesterase and serum cholinesterase are decreased in patients with pernicious anemia in relapse and that the activity of this enzyme returns to normal with the institution of adequate therapy. Glob and associates<sup>3</sup> administered DFP (diisopropyl fluorophosphate) to two patients with pernicious anemia and induced a complete remission with folic acid and liver therapy in spite of sustained suppression of the plasma and red cell esterase activity caused by the daily administration of DFP. Other investigators have noted that the maintenance of low red cell and plasma esterase activity in man<sup>4</sup> and animals<sup>5</sup> following repeated injections of DFP produced no changes in hepatic, renal or hematologic functions.

Many attempts have been made to correlate the enzymic activity of cholinesterase with myasthenia gravis,<sup>6</sup> liver disease,<sup>7</sup> psychiatric disorders,<sup>8</sup> hypertension,<sup>9</sup> the allergic states,<sup>10</sup> and endocrine dysfunction.<sup>11</sup>

Barnard<sup>12</sup> has stated his belief that individual reactivity to a stimulant or inhibitor with the production of excessive cholinesterase on the one hand, or deficient enzyme on the other, determines the onset and type of pathologic and malignant hematopoietic disease. Davis<sup>13, 14</sup> postulates that the presence of low blood cholinesterase and the associated high acetylcholine concentration is the responsible mechanism for pernicious anemia in relapse.

We have accumulated data of the activity of red cell esterase and serum esterase of patients with various types of leukemia, with and without anemia, various primary and secondary anemias, polycythemia, and myelophthisis in a further effort to clarify the role of cholinesterase found in the red blood cell.

## METHODS AND MATERIALS

The cholinesterase activity of whole blood and serum was determined in 101 patients and twenty one normal subjects, adults and children of both sexes and all races. These determinations were made from random samples drawn at odd times of day and night. In all but the polycythemic patients, therapeutic procedures, blood transfusions, and radiation therapy were avoided prior to the blood sampling. The control series of twenty one normal subjects included fifteen subjects previously reported.<sup>15</sup> Values for ten of the eleven patients with pernicious anemia in relapse and nine of the ten patients in remission have been reported.<sup>2</sup>

Venous blood was drawn from the antecubital vein with a dry syringe and needle. Then 5 ml of whole blood were oxalated with anticoagulant mixture (Wintrobe), and 5 ml were collected in a dry test tube and allowed to clot. The hematocrit (Wintrobe) was determined and a complete capillary blood count done. Reticulocyte counts were also made on some of the more anemic patients.

Cholinesterase activity was measured by the method described in a previous report.<sup>15</sup>

From the Department of Therapeutics, New York University College of Medicine.  
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TABLE I THE MEAN VALUES OF CHOLINESTERASE ACTIVITY OF THE SERUM AND RED CELLS IN VARIOUS HEMATOLOGIC DISORDERS

DISEASES	NUMBER IN GROUP	HB (GM / 100 ML)	RBC ( $\times 10^6$ / MM <sup>3</sup> )	MCV (CU $\mu$ )	MC HB ( $\gamma\gamma$ )	CHOLINESTERASE	
						SERUM (UNITS / ML)	MEAN CELL (UNITS $\times 10^{10}$ PER CELL)
Normal	21	14	5.20	85	29	3.22	7.57
P.A. in relapse	11	3.3	1.0	122	42	1.62	6.05
P.A. in remission	10	14.9	4.60	87	30	2.61	8.74
Chr lymph leucemia	24	12	97	95	31	2.38	8.67
Chr myel leucemia	11	10.1	52	92	29	2.34	0.60
Acute leucemia	-	11	10	85	31	1.79	5.1
Myelophthisis	-	12	187	93	34	1.99	5.97
Hodgkin's disease	7	12	4.04	92	30	2.64	7.60
Polythemia vera	10	16.5	6.24	95	27	3.08	7.70
Secondary anemias	13	10.4	64	87	29	2.20	8.94
Sickle cell anemia	5	10	6.8	105	26	3.99	9.84

MCV Mean corpuscular volume in cubic microns

MC HB Mean corpuscular hemoglobin in micromicrograms

## RESULTS

The blood of twenty-four patients diagnosed as having chronic lymphocytic leucemia was studied. Table II demonstrates the range of hemoglobin (6.5 to 15.0 Gm per cent) and red cell values (2.5 to 6.3 M). In general these patients were in good clinical condition. The bone marrow was infiltrated with great numbers of lymphocytes and the extramedullary tissue was reduced in quantity. The mean cell hemoglobin concentration and the mean cell volume were within the normal range. The cholinesterase activity of the serum varied from 0.85 to 3.64 units and reflected the clinical condition of the patient. The mean serum esterase of the entire group was 2.38 units as compared with a normal of 3.2 units. The mean corpuscular esterase activity for the entire group was 8.67 units ( $\times 10^{10}$ ), compared with 7.57 units ( $\times 10^{10}$ ) in the normal. In this group, therefore, despite the presence of a mild anemia the mean cholinesterase activity of the red cell esterase was within upper normal limits.

Blood samples of eleven patients with chronic myelocytic leucemia were studied. Table III shows the range of hemoglobin (4.5 to 13.5 gm per cent) and red cell (1.5 to 5.15 M) values. The general condition of this group was good but these patients were sicker than those with lymphocytic leucemia. The mean corpuscular hemoglobin and the mean cell volume were normal. The activity of the serum esterase varied from 1.00 to 4.62 with a mean value of 2.34 units, which further reflected the general condition of this group. The mean corpuscular esterase activity varied from 4.22 to 11.25 units ( $\times 10^{10}$ ) with a mean value of 6.60. This value is not significantly lower than the normal mean of 7.57 units.

Studies of the blood of seven patients with acute leucemia were made (Table IV). Of these, five were myeloblastic and two, lymphoblastic. The hemoglobin varied from 3.5 to 11.5 Gm per cent and the red count ranged from 1.0 to 4.4 million per cubic millimeter. The serum esterase values were low.

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## METHODS AND MATERIALS

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Cholinesterase activity was measured by the method described in a previous report.<sup>15</sup>

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TABLE IV  
CHOLINESTERASE ACTIVITY OF THE SERUM AND RBC CELLS IN ACUTE LEUCEMIA[illegible]

Patients and 6 were diagnosed as having acute lymphoid leucemia the remainder as having acute myeloid leucemia

Three patients satisfying the criteria for a diagnosis of myelophthisic anemia exhibited macrocytosis and a significantly low red cell esterase activity of 5.97 units ( $\times 10^{-10}$ ). The serum esterase was markedly reduced to 1.99 units (Table 3).

TABLE 1 CHOLINESTERASE ACTIVITY OF THE SIVM AND RBC CELLS IN MYELOPHTHISIC ANEMIA

PATIENT	HB (GM / 100 ML.)	FBC ( $\times 10^6$ / MM <sup>3</sup> )	FETIC ( $\times 10^3$ / MM <sup>3</sup> )	MCV (CU $\mu$ )	MCHB (GG)	CHOLINESTERASE	
						SERUM (UNITS / ML.)	MEAN CELL (UNITS $\times 10^{10}$ / PEP CELL)
1	7.00	20.	61	98	34	1.64	6.38
2	6.00	1.82		94	33	1.36	5.77
3	5.70	1.64		81	35	2.96	5.76
Mean	6.25	1.8		92	34	1.99	5.91
						Standard deviation = $\pm 0.35$	

The blood of seven patients with Hodgkin's disease and ten patients with polycythemia vera was studied. In the former group (Table VI) a mild normochromic normocytic anemia was found. The red cell esterase activity 7.60 units ( $\times 10^{10}$ ), compared favorably with normal values. The serum esterase activity 2.64 units was only slightly depressed.

TABLE VI CHOLINESTERASE ACTIVITY OF THE SERUM AND RED CELLS IN HODGKIN'S DISEASE

[illegible]

The blood findings in the polycythemic group (Table VII) showed the characteristic high hemoglobin content and normocytic to microcytic sized cells, but again the cholinesterase activity of both the serum, 3.08 units, and the cells, 7.70 units ( $\times 10^{-10}$ ), was almost identical with that found in normal subjects

TABLE VII CHOLINESTERASE ACTIVITY OF SERUM AND RED CELLS IN POLYCYTHEMIA VPPA

PATIENT	HB (GM / 100 ML)	R.B.C ( $\times 10^6$ PER MM <sup>3</sup> )	M.C.V. (CU $\mu$ )	M.C.H.B. ( $\gamma\gamma$ )	CHOLINESTERASE	
					SERUM (UNITS ML)	MEAN CELL (UNITS $\times 10^{-10}$ PER CELL)
1	18.0	7.40	90	24	4.58	8.57
2	17.4	5.50	99	32	4.12	10.25
3	14.5	7.20	77	20	3.18	10.14
4	18.9	7.30	84	27	3.40	8.34
5	13.6	4.80	85	29	3.54	6.66
6	15.7	4.80	89	29	2.19	7.81
7	11.8	4.50	84	26	3.41	5.62
8	21.5	8.40	82	26	3.01	6.78
9	13.6	5.50	76	26	1.87	6.98
10	20.0	7.00	83	29	1.52	5.92
Mean	16.5	6.24	85	27	3.08	7.70

Standard deviation =  $\pm 1.56$ 

TABLE VIII CHOLINESTERASE ACTIVITY OF SERUM AND RED CELLS IN VARIOUS SECONDARY ANEMIAS

PATIENT AND DISEASE	HB (GM PER 100 ML)	R.B.C ( $\times 10^6$ PER MM <sup>3</sup> )	RETIC ( $\times 10^3$ PER MM <sup>3</sup> )	M.C.V. (CU $\mu$ )	M.C.H.B. ( $\gamma\gamma$ )	CHOLINESTERASE	
						SERUM (UNITS / ML)	MEAN CELL (UNITS $\times 10^{-10}$ PER CELL)
1 Gastric cancer	6.5	1.90	57	89	33	1.10	8.63
2 Bronchogenic cancer	12.5	3.50		91	36	2.42	10.08
3 Seminoma testicle	7.5	3.20		75	23	0.40	6.00
4 Lupus erythematosus	10.0	3.40	41	76	29	2.41	7.36
5 Arterioscl heart dis	11.0	4.52		80	24	1.82	9.40
6 Simmonds' disease dis	13.3	4.01	32	95	33	3.07	11.76
7 Hypertens heart dis	8.0	3.20	22	84	25	2.48	8.09
8 Rheumatic heart dis	13.5	5.08		80	26	2.88	8.23
9 Arterioscl heart dis	12.5	3.45		110	36	4.56	8.84
10 Rheumatic heart dis	13.5	4.30		103	31	2.40	9.93
11 Diverticulitis colon	7.5	2.78	56	89	27	2.06	9.39
12 G.I. Bleeding site ?	9.0	3.72	147	78	25	1.42	9.88
13 G.I. Bleeding site ?	10.7	4.30		86	25	1.52	8.78
Mean	10.4	3.64		87	29	2.20	8.94

Standard deviation =  $\pm 1.39$

In order to study the effect of active erythropoiesis upon cholinesterase activity two groups of patients were tested. In the first group thirteen patients with secondary anemias (Table VIII) of a hypochromic normocytic type exhibited a red cell esterase activity of 8.94 ( $\times 10^{-10}$ ) units as compared with 7.57 in the normal subjects. The serum esterase of these subjects was depressed. Since many of these patients were debilitated this later finding was anticipated. A second group of five patients with sickle cell anemia in relapse (Table IX) was examined. All five showed active reticulocytosis. In all, the mean cell volume and mean cell hemoglobin were above normal. The serum cholinesterase values were normal. The red cell esterase activity however was markedly increased and a mean volume of 9.40 units ( $\times 10^{-10}$ ) was obtained.

TABLE IX. CHOLINESTERASE ACTIVITY OF SERUM AND RED CELLS IN SICKLE CELL ANEMIA IN REMISSION

PATIENT	HB (GM / 100 ML.)	R.B.C. ( $\times 10^6$ PFC / MM <sup>3</sup> )	RETIC. ( $\times 10^3$ / MM <sup>3</sup> )	MCV (CU $\mu$ )	MCV HB (G%)	CHOLINESTERASE	
						SERUM (UNITS / ML.)	MEAN CELL (UNITS $\times 10^{10}$ / PFC CELL)
1	9.5	2.20	330	100	22	3.32	11.54
2	7.9	2.80	280	96	27	3.34	9.86
	9.0	2.20	3.0	17	9	3.00	9.11
4	12.0	4.00	100	88	30	3.42	8.40
5	9.0	2.20	198	109	24	3.10	10.31
Mean	9.48	2.68	268	105	26	3.29	9.40

Standard deviation =  $\pm 1.10$

#### DISCUSSION

Grob, Lilienthal and co workers<sup>1</sup> have recently reported that following the administration of DFP to normal and anemic subjects the red blood cell esterase regenerated at a rate approximating the calculated rate of red cell replacement in the body and was proportional to the number of circulating reticulocytes. The observed daily cholinesterase regeneration rate was 12 per cent. This agrees closely with the calculated daily erythrocyte replacement rate of 0.83 per cent which is based on a red cell life span of 120 days. Koelle and Gilman,<sup>4</sup> working with experimental animals observed similar findings. In a study<sup>2</sup> of the cholinesterase activity of patients with pernicious anemia in relapse who were followed into remission, we found that the rise in cholinesterase activity of the whole blood was primarily due to a rise in activity of the red cell itself. We further reported that this increased activity could not be correlated to cell size nor to the presence of reticulocytosis per se. The mean value for any one red cell was found to vary from low values in patients in relapse to above normal values in early remission. Normal values were obtained only seventy to ninety days following the institution of specific therapy.

The present study substantiates our previous report. Nevertheless Table I demonstrates that increases in cholinesterase activity of the red blood cells is noted in those diseased states where increased erythropoietic activity is present, that is, the secondary anemias sickle cell anemia in relapse, and pernicious



anemia in early remission Table I also shows that there is some increase in the red cell esterase activity of patients with chronic lymphocytic leukemia, although the bone marrow of the majority of these patients was overwhelmingly infiltrated with lymphocytes and the quantity of erythroid tissue markedly reduced The physiologic implication of this is not yet clear

Conversely, those diseases not associated with increased erythropoiesis and those in which erythropoiesis is depressed show significant depression of red cell esterase activity

A variety of liver function tests made from a sampling of patients with chronic lymphocytic leukemia, chronic myelocytic leukemia, pernicious anemia in relapse, and the secondary anemias did not reveal any consistent correlation to the red cell cholinesterase activity The total protein and albumin concentrations of the serum tended to parallel the serum esterase activity

Although we are unable to explain our findings, we feel that the cholinesterase activity of the red cells as measured in the regenerative anemias is not merely a reflection of the immature cells released into the peripheral blood, but is associated in some other manner with increased red cell production

#### SUMMARY

The cholinesterase activity of the serum and red blood cells of 101 patients with various hematologic diseases was studied and compared with values found in twenty-one normal subjects

The red blood cells of patients with pernicious anemia in early remission, sickle cell anemia, and hypochromic normocytic anemias manifested a cholinesterase activity greater than that of the normal erythrocyte

The red blood cells of patients with pernicious anemia in relapse, acute leukemia, and myelophthisic anemia showed depression of cholinesterase activity

Patients with chronic myelocytic leukemia, Hodgkin's disease, and polycythemia vera were noted to have erythrocytes with normal cholinesterase activity

The mean red cell esterase activity of patients with chronic lymphocytic leukemia was increased to a high normal level

The authors wish to acknowledge the many helpful criticisms and suggestions of Dr Howard M Fitch, Department of Therapeutics, New York University College of Medicine

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# IN VITRO EFFECTS ON GRAM-NEGATIVE BACTERIA OF STREPTOMYCIN COMBINED WITH PENICILLIN AND/OR SULFADIAZINE

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THE therapeutic value of streptomycin is now well established in the treatment of many types of infectious disease of gram-negative and acid-fast etiology<sup>1, 2, 3</sup>. More general use of this agent has, however, been curbed by two important limitations: (1) rapid development of microbial resistance to the drug, and (2) toxicity following prolonged administration with full doses.

The studies reported in this communication were designed to find a solution to these limitations, with particular emphasis on drug fastness. The selective overlapping antibacterial spectra of penicillin, sulfonamides, and streptomycin,<sup>4</sup> and the demonstration by Carpenter<sup>5</sup> and others<sup>6</sup> of the suppression of drug-resistant mutants when certain of these agents are used in combination, served as a background. The plan of the investigation included (1) a study of the comparative bacteriostatic action in vitro of streptomycin and of five purified fractions of penicillin against gram-negative bacteria, and (2) a study of the comparative rate of development of streptomycin fastness of gram-negative bacteria (a) to streptomycin alone, to streptomycin in combination with (b) penicillin and (c) sulfadiazine, and (d) to mixtures of all three agents.

## STREPTOMYCIN AND PENICILLIN

*Materials and Methods*—Streptomycin hydrochloride (Merck) and sodium sulfadiazine were obtained in the open market. The purified penicillin fractions F, dihydro F, X, and K were kindly supplied by the National Institute of Health.

Ammonium penicillin F Upjohn, Lot No. 123—BEL—4, 1625 U/mg

Sodium penicillin dihydro F Pfizer, Lot No. 160619, 1610 U/mg

Ammonium penicillin X Commercial Solvents Corporation, Lot No. 150268, 850 U/mg

Sodium penicillin K Pfizer, 7/12/46, 2075 U/mg

A supply of purified crystalline sodium penicillin G, Lot No. 104115, was furnished through the courtesy of Commercial Solvents Corporation.

All agents used in this investigation were prepared on a weight basis by dilution in triple distilled water. The test organisms were maintained on infusion agar under oils and were grown in test broth (Food and Drug Administration) prior to the experiments. The double strength FDA broth employed has the formula: 2 per cent peptone, 1 per cent beef extract, and 0.5 per cent sodium chloride, in water, adjusted to pH 7.2. The single strength broth contains half this concentration.

The procedure of testing was as follows. One milliliter of stock antibiotic solutions progressively diluted in sterile distilled water was added to a series of sterile tubes. The tubes were inoculated with 1 ml. of 1:50 dilution of a six-hour growing culture of the test organism in double strength broth. The end point was read at the lowest concentration showing no turbidity after forty hours' incubation at 37° C.

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**Results**—All the bacteria listed in Table I were tested simultaneously to penicillin G and to streptomycin. Because of the limited supply of penicillins only representative members of each species were tested also against the purified fractions F, dihydrio I, X and K. The readings listed in Table I indicate the highest dilution in micrograms per milliliter of penicillin and of streptomycin which showed no growth at the end of forty hours incubation. For purposes of discussion, cultures completely inhibited by 10  $\mu$ g per milliliter or less of antibiotic are classified as sensitive while all others are classified as resistant.

It will be noted that penicillinate bacteria exhibit a wide variation in degree of sensitivity to the purified penicillins. Generally penicillin G was as effective as or more effective than the X dihydrio I and F fractions. A constant finding was the relative inferiority of penicillin K in comparison with the other fractions.

All five strains of *Neisseria facialis* and seven of twelve strains of *Brucella*\* were extremely sensitive to penicillin G. The superiority of penicillin X against *Brucella* over the other fractions is noteworthy: all twelve strains were completely inhibited by 14  $\mu$ g per milliliter or less. This has been shown to be a bacteriostatic effect lasting through 120 hours but not bactericidal at the time of the forty hour reading. Five of eleven *Proteus* strains and five of twelve *Salmonella* strains were inhibited by concentrations below 10  $\mu$ g per milliliter of penicillin G. The remaining *Salmonella* organisms were moderately resistant while all but two of the remaining *Proteus* strains were very resistant. Some strains among the *Proteus* and *Salmonella* species were more sensitive to penicillin G than to streptomycin microgram and microgram and vice versa. The action of penicillins X and C on *Enteriella* is noteworthy. Nine of ten strains were inhibited in the range of 4 to 20  $\mu$ g per milliliter. The tenth strain was very resistant. All but one of ten *Shigella* strains were refractory to 10  $\mu$ g per milliliter penicillin G and this group in general was found to be moderately resistant. *Escherichia coli*, the *Aerobacter* Friedlander group and *Pseudomonas* species were very resistant to penicillin especially *Pseudomonas*. The survey suggests that gram negative bacteria show broader range of reaction to penicillin than to streptomycin. The superiority of streptomycin activity over penicillin against gram negative bacteria was reaffirmed. All nine genera showed strains sensitive to streptomycin, though some moderately resistant strains occurred among *Salmonella*, *Proteus* and, predominantly, *Pseudomonas*.

#### STREPTOMYCIN, PENICILLIN, AND SULFADIAZINE

**Materials and Methods**—The demonstration of wide generic and species variation in sensitivity to penicillin, and of the greater sensitivity of some organisms to penicillin than to streptomycin led to the second phase of this study. This phase was concerned with the effects of combinations of penicillin G, sodium sulfadiazine and streptomycin on the sensi-

\*These *Brucella* were not freshly isolated organisms but stock cultures obtained (in order of presentation in Table I) from the following sources: Dr. Schuchardt, University of Texas Nos. 4, 6, 8, 9, 10 and 11; Col. D. Kuhns, 4th Army Medical Laboratory Nos. 1, 3 and 5; and Dr. H. L. Morton, University of Pennsylvania School of Medicine Nos. 1 and 5. Penicillin is bacteriostatic not bactericidal. Growth is inhibited by the concentrations listed for longer than five days. Addition of penicillin to tubes showing complete inhibition causes regrowth of the organisms; the rate of regrowth being inversely proportional to the concentration of penicillin in which they were exposed.

TABLE I COMPARATIVE IN VITRO ACTION OF PURIFIED PENICILLIN FRACTIONS AND STREPTOMYCIN ON GRAY NEGATIVE BACTERIA

ORGANISM	PENICILLIN ( $\mu\text{G}/\text{ML}$ )				STREPTOMYCIN ( $\mu\text{G}/\text{ML}$ )	
	G	$\lambda$	DP	F	K	
Aerogenes Friedlander species	50	175	75	250	100	8
Aerogenes Friedlander species	>250	>250	>250	>250	>250	4
Aerogenes Friedlander species	>250	>250	>250	>250	>250	2
Aerogenes Friedlander species	250					2
Aerogenes Friedlander species	>250					8
Aerogenes Friedlander species	>250					8
Alcaligenes faecalis	0.6					<0.5
Alcaligenes faecalis	0.6					<0.5
Alcaligenes faecalis	1.2		2.5	2.5	5	16
Alcaligenes faecalis	5.0					4
Brucella abortus	0.44					0.5
Brucella abortus	0.6	0.22	0.3	0.3	0.07	4
Brucella abortus	0.88	0.3				1
Brucella abortus	0.88	0.44				<0.1
Brucella abortus	1.2	0.3	0.3	2.5	12.5	1
Brucella abortus	7	1.75				<0.1
Brucella abortus	7	0.88				<0.1
Brucella abortus	28	3.5				<0.1
Brucella abortus	>28	7				<0.1
Brucella abortus	>28	7				<0.1
Brucella abortus	>28	14				<0.1
Brucella abortus	31	2				<0.1
Brucella abortus	4	5	9	9	75	1
Brucella abortus	8					8
Brucella abortus	8					16
Brucella abortus	9					8
Brucella abortus	12	12	9	12	100	4
Brucella abortus	12	12	2.5	19	75	8
Brucella abortus	12	12	19	35	175	2
Brucella abortus	12	12	35	75	250	1
Brucella abortus	19	19	25	25	100	4
Brucella abortus	19	19	35	100	250	16
Brucella abortus	>250					125
Brucella abortus	35	175	50	100	250	8
Brucella abortus	35	175	75	175	175	4
Brucella abortus	75	100	175	>250	>250	8
Brucella abortus	100	175	100	>250	>250	8

[illegible]

tivity and on the rate of development of resistance to streptomycin of five gram negative bacteria in the presence of combinations of the drug. The comparative sensitivities in micrograms per milliliter of the test organisms to the two antibiotics are as follows:

	STREPTOMYCIN	PENICILLIN
<i>Proteus vulgaris</i>	32	12
<i>Proteus</i> OX2	4	250
<i>Proteus</i> X19	16	>125
<i>Escherichia coli</i>	8	35
<i>Pseudomonas aeruginosa</i>	16	5,000

The object of the first experiment was to determine whether penicillin or sulfadiazine would prevent or delay the emergence of streptomycin fast bacteria. The concentration of penicillin, 15  $\mu$ g per milliliter, was the constant and the concentrations of streptomycin were the variables. All strains grew freely in broth containing this concentration of penicillin. A parallel experiment was set up to determine whether sulfadiazine, 150  $\mu$ g per milliliter, would prevent or delay drug fastness to streptomycin when the two drugs were present in combination. Bacteria grew freely in broth containing this amount of sodium sulfadiazine.

TABLE II. EFFECTS OF PENICILLIN, SULFADIAZINE ALONE AND IN COMBINATION ON SENSITIVITY AND FASTNESS OF BACTERIA TO STREPTOMYCIN

	P. VULGARIS		P. OX2		P. X19		ESCH. COLI		PS. AERUGINOSA	
	A	B	A	B	A	B	A	B	A	B
Streptomycin (S)	32	6	4	4	16	5	8	13	16	6
S and P*	2	13	1	4	16	4	8	13	16	6
S and SAD†	8	6	1	10	2	43	8	28	8	8
S and P‡ and SAD§	0.5	20	1	7	2	30	4	24	8	14

A Streptomycin concentration required to inhibit growth

B Number of daily transfers in streptomycin to effect drug fastness

\*P Concentration of penicillin 15  $\mu$ g/ml

†SAD Concentration of sodium sulfadiazine 150  $\mu$ g/ml

‡P Concentration of penicillin 0.1 to 0.75  $\mu$ g/ml (see text)

§SAD Concentration of sodium sulfadiazine 10 to 75  $\mu$ g/ml (see text)

The experiments were conducted as follows. Serial dilutions of streptomycin in the range of 256 to 0.5  $\mu$ g were prepared in 1 ml amounts, and 0.1 ml of the adjuvant solution (penicillin or sulfadiazine) was added to give the desired final concentration. The tubes were inoculated with 1.1 ml of 1:50 dilution in double strength broth of a six hour growing culture of the test organism (estimated three hundred million organisms per milliliter). They were incubated for fifteen to eighteen hours at 37° C and then read. The organisms showing growth in the tube containing the highest concentration of streptomycin were seeded to normal broth and allowed to grow for approximately six hours. The test was then repeated with this culture. This procedure was continued daily until the organisms showed consistent streptomycin resistance. For controls, one series with streptomycin alone was transferred each day until drug fastness occurred. To check the potency of the antibiotics, a test was set up daily with organisms of known sensitivity. Platings at intervals through each series checked for purity of culture.

The object of the second experiment was to determine whether penicillin and streptomycin together with sodium sulfadiazine would prevent or delay the emergence of streptomycin fast bacteria. When both penicillin and sodium sulfadiazine were combined in half the concentration previously employed, in every instance, except that of *Pseudomonas*, there was complete bacteriostasis even in the lowest concentration (0.5  $\mu$ g per milliliter) of streptomycin. Consequently, the concentrations of penicillin and of sulfadiazine had to be reduced two to eight times to permit growth in the presence of mixtures of the three agents.

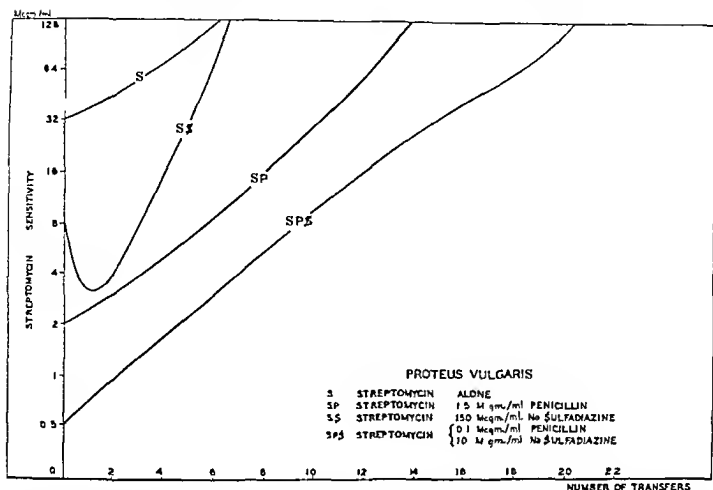


Fig 1—Demonstrates the effect of combinations of penicillin and/or sodium sulfadiazine on streptomycin sensitivity and rate of developing streptomycin fastness of *P. vulgaris*. The ordinates show the amount of streptomycin in micrograms per milliliter required to produce complete bacteriostasis when used singly when combined with either penicillin or sodium sulfadiazine and when the three agents were employed together. The number of daily transfers required for streptomycin resistance to occur under conditions of the test is indicated on the abscis axis.

**Results**—The results of the *in vitro* effects of combinations of penicillin and sulfadiazine with streptomycin are shown in Figs 1 to 5 and in Table II. As shown in Fig 1 and Table I *P. vulgaris* required 32 and 12  $\mu$ g per milliliter of streptomycin and penicillin G respectively to effect complete bacteriostasis. Six transfers in streptomycin broth were required for the organisms to grow freely in excess of 100  $\mu$ g streptomycin per milliliter. The addition of 15  $\mu$ g per milliliter of penicillin G reduced the streptomycin requirement from 32 to 2  $\mu$ g per milliliter, which represents one sixteenth and one eighth of the individual requirement of the two substances. The penicillin streptomycin combination delayed the time of occurrence of drug resistance from six to thirteen transfers. Sodium sulfadiazine in the amount of 150  $\mu$ g per milliliter reduced the bacteriostatic concentration of streptomycin from 32 to 8  $\mu$ g per milliliter, which is a fourfold reduction. Sodium sulfadiazine however, did not alter the rate of emergence of streptomycin resistant organisms. Penicillin and sodium sulfadiazine combined in one quarter amounts (0.37 and 37  $\mu$ g per milliliter respectively) allowed growth of this organism, but the addition of only 0.25  $\mu$ g per milliliter streptomycin to this combination was completely inhibitory. The combination of penicillin and sodium sulfadiazine in only one eighth the amounts used alone reduced the streptomycin requirement from 32 to 0.5  $\mu$ g per milliliter, a sixty four fold decrease. A synergistic action of these three drugs on this organism is suggested. Moreover with this combination, drug fastness to streptomycin was retarded from six to twenty transfers. It is significant that drug resistance to streptomycin ultimately did occur.



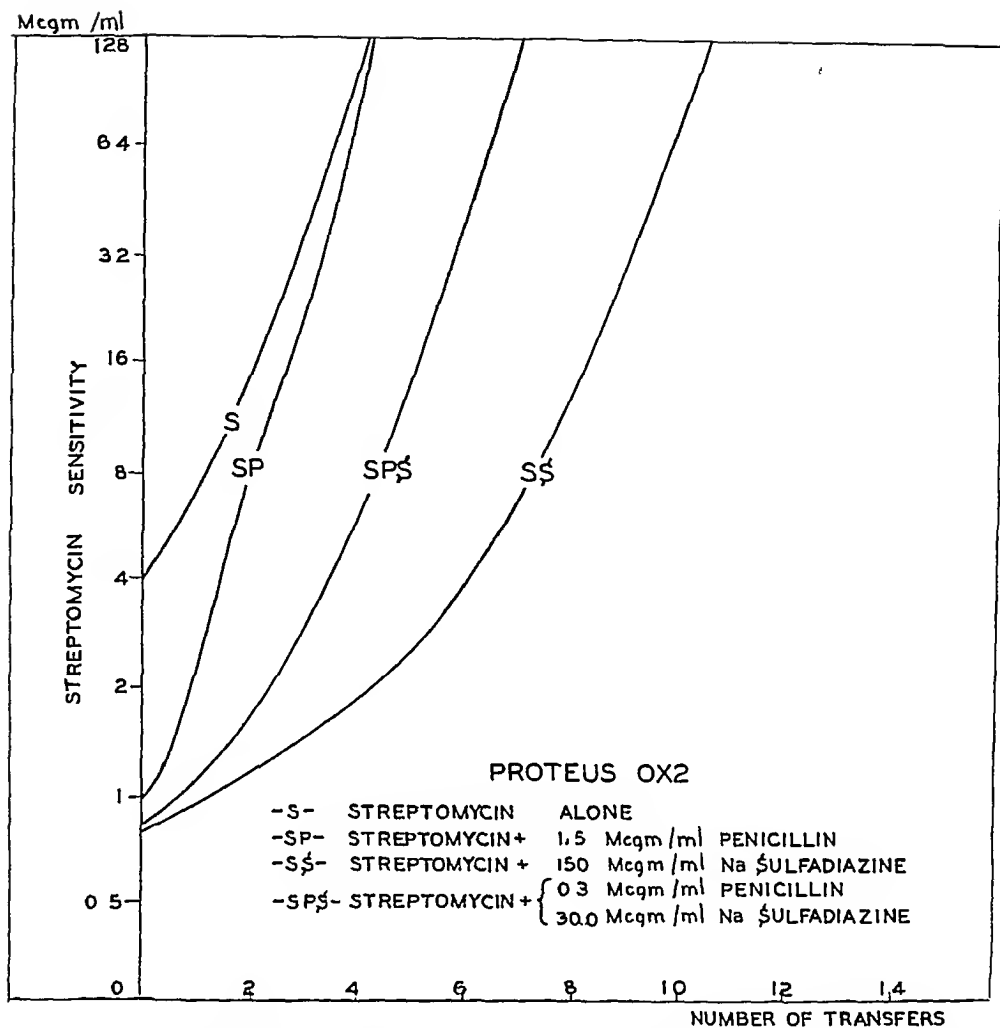


Fig 2—Demonstrates the effects on streptomycin sensitivity and rate of development of streptomycin fastness of *Proteus* OX2 when streptomycin is combined with penicillin and/or sodium sulfadiazine

Fig 2 illustrates the results with a strain of *Proteus* OX2. This culture required 4  $\mu$ g per milliliter streptomycin or 250  $\mu$ g per milliliter penicillin for complete suppression. The organism became fast to 100  $\mu$ g per milliliter after four transfers in streptomycin broth. The addition of 15  $\mu$ g per milliliter of penicillin G reduced the streptomycin requirement from 4 to 1  $\mu$ g per milliliter, in the face of apparent penicillin resistance of the organism. This combination effected no delay in the time of acquired resistance. Sodium sulfadiazine, 150  $\mu$ g per milliliter, reduced the streptomycin requirement from 4 to 1  $\mu$ g per milliliter, and with this combination ten transfers were required to produce a streptomycin-fast culture against four transfers with streptomycin alone. The simultaneous combination of penicillin and sodium sulfadiazine with streptomycin in one-fourth the concentrations used individually also effected a reduction in the streptomycin requirement from 4 to 1  $\mu$ g per milliliter, and delayed development

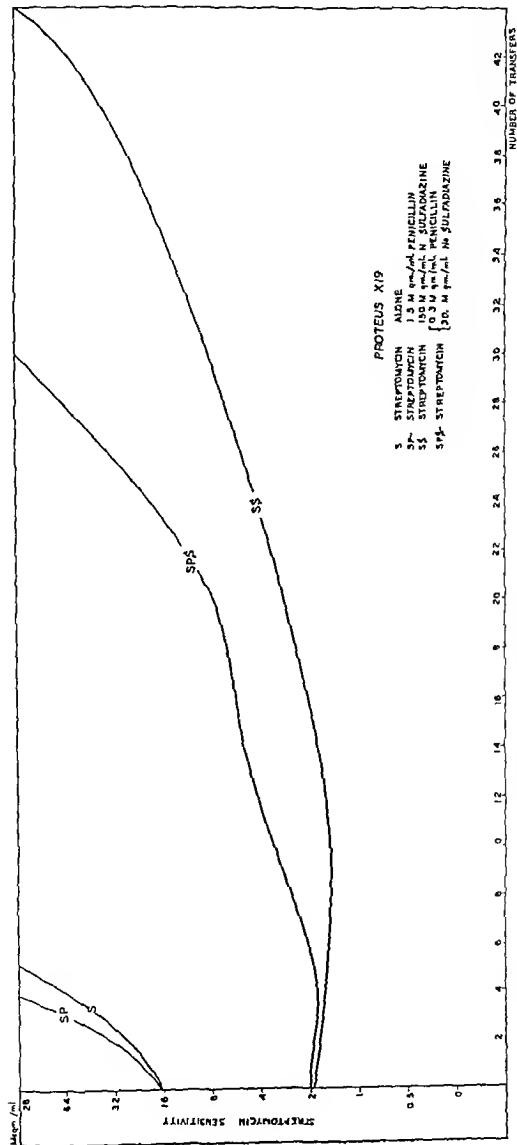


Fig 3—Demonstrates the effects on streptomycin sensitivity and rate of development of streptomycin fastness of *Proteus* X19 when streptomycin is combined with penicillin and/or sodium sulfadiazine

of streptomycin fastness from four to seven transfers. Although this result represents a less impressive effect than that achieved with sodium sulfadiazine and streptomycin, it is emphasized that only one-fourth the concentrations of sulfadiazine and penicillin were used. Concentrations in excess of this, when combined with the 0.5  $\mu\text{g}$  streptomycin per milliliter, caused complete inhibition.

Fig. 3 represents the effects of the various agents on streptomycin sensitivity of a strain of *Proteus* X19. This organism required 16  $\mu\text{g}$  per milliliter streptomycin and greater than 125  $\mu\text{g}$  per milliliter penicillin G individually for complete inhibition. With streptomycin alone, five transfers in sub-bacteriostatic

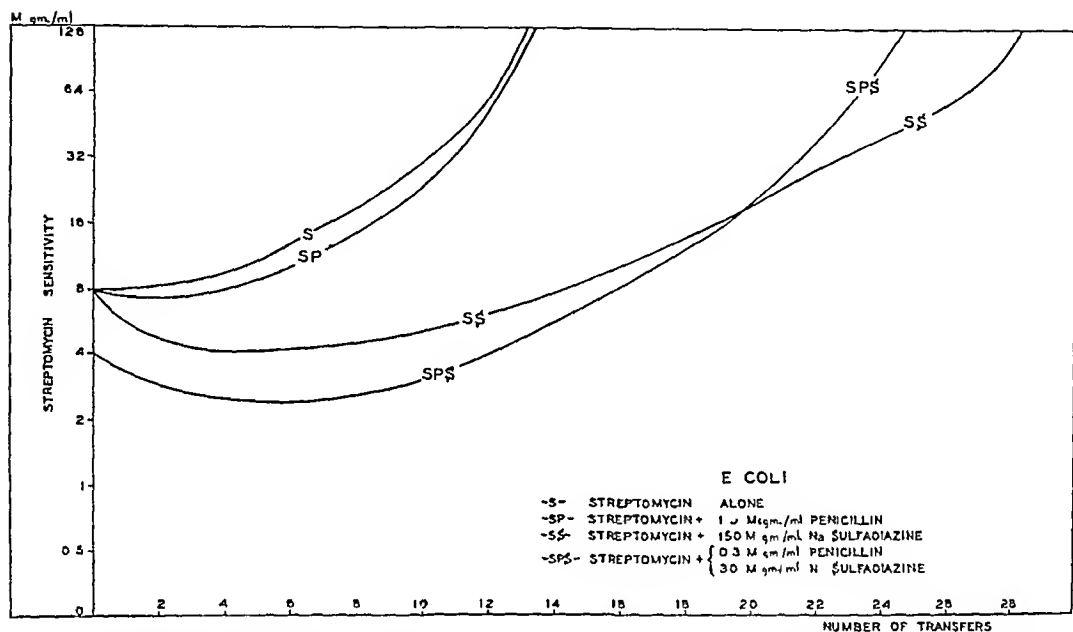


Fig. 4—Demonstrates the effects on streptomycin sensitivity and rate of development of streptomycin fastness of *Esch. coli* when streptomycin is combined with penicillin and/or sodium sulfadiazine.

concentrations were made before streptomycin fastness to 100  $\mu\text{g}$  per milliliter was attained. The addition of 15  $\mu\text{g}$  per milliliter penicillin G reduced neither the streptomycin requirement nor the number of transfers necessary for resistance to occur. Sodium sulfadiazine effected a reduction of streptomycin requirement from 16 to 2  $\mu\text{g}$  per milliliter and the striking resistance delay from five to forty-three transfers. The mixture of penicillin G and sodium sulfadiazine in the media in one-fourth the individual concentrations reduced the streptomycin requirement again from 16 to 2  $\mu\text{g}$  per milliliter and effected a delay in resistance to streptomycin from five to thirty transfers. In this instance also, the use of concentrations in the mixture in excess of one-fourth each with 0.5  $\mu\text{g}$  per milliliter streptomycin prevented growth.

Fig. 4 illustrates the effects of the combined drugs on *Esch. coli*. Streptomycin 8  $\mu\text{g}$  per milliliter and penicillin G 35  $\mu\text{g}$  per milliliter individually completely inhibited this organism. In streptomycin broth, thirteen transfers were

made before the culture showed consistent growth in 100  $\mu\text{g}$  per milliliter. The addition of 15  $\mu\text{g}$  per milliliter penicillin G caused neither reduction of streptomycin requirement nor delay in acquired resistance. Sodium sulfadiazine 150  $\mu\text{g}$  per milliliter caused no decrease in initial streptomycin requirement but effected a delay in acquired resistance from thirteen to twenty eight transfers.

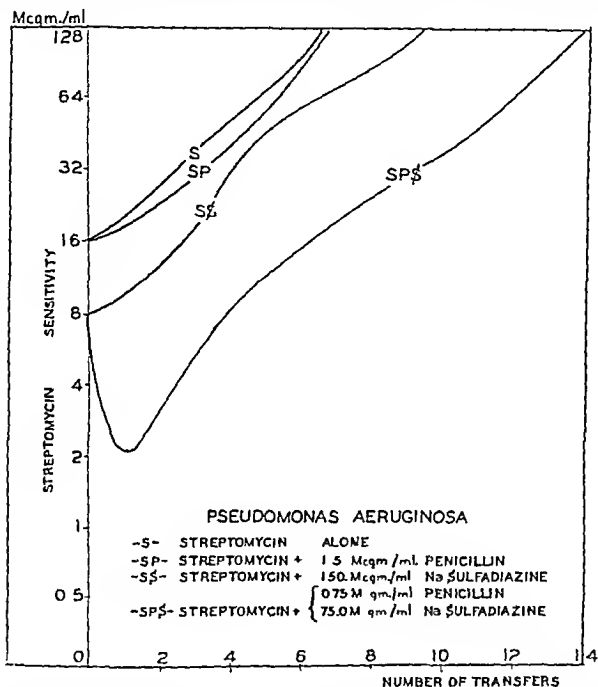


Fig 5—Demonstrates the effect on streptomycin sensitivity and rate of development of streptomycin fastness of *Ps aeruginosa* when streptomycin is combined with penicillin and/or sodium sulfadiazine

One half these amounts of penicillin G and sodium sulfadiazine with 0.5  $\mu\text{g}$  per milliliter streptomycin allowed no growth. When one fourth the individual concentrations were used together, the streptomycin needed for complete inhibition was reduced from 8 to 4  $\mu\text{g}$  per milliliter and resistance was delayed from thirteen to twenty four transfers.

Fig 5 demonstrates the effects of combined antibacterial agents on *Ps aeruginosa*. This strain required 16  $\mu\text{g}$  streptomycin per milliliter and 5 000  $\mu\text{g}$  per milliliter penicillin G individually for complete bacteriostasis. With streptomycin alone, six transfers occurred before resistance was acquired. Penicillin

15  $\mu\text{g}$  per milliliter had no effect on the necessary initial concentration of streptomycin nor rate of emergence of resistance. Sodium sulfadiazine 150  $\mu\text{g}$  per milliliter reduced the initial streptomycin requirement from 16 to 8  $\mu\text{g}$  per milliliter and delayed resistance from six to eight transfers only. However, when one-half the concentrations of penicillin G and sodium sulfadiazine used individually were combined with streptomycin, there was a delay in acquired resistance from six to fourteen transfers. When the combination of these three drugs was tested in the same manner against eight additional strains of *Pseudomonas*, similar results were obtained with five. Three strains initially resistant to streptomycin evidenced no effect by the addition of the other agents within the range of the experiment.

#### DISCUSSION

Restudy of the action of penicillin on gram-negative bacteria was basic to investigation of the possibility of suppressive effects when the antibiotic was combined with streptomycin. The susceptibility to penicillin G and X of *Neisseria*<sup>9</sup> and *Hemophilus*<sup>10a</sup> is well known. Survey of nine other genera of gram-negative organisms indicated that *Alcaligenes* and *Brucella*, in the main, were moderately sensitive. *Eberthella*<sup>10b</sup> was slightly sensitive. On the other hand, *Pseudomonas*, *Aerogenes*, *Friedlander*, *Shigella*, and *Escherichia* were moderately to completely resistant. Several strains of *Proteus* and *Salmonella* were more susceptible to penicillin than to streptomycin, but the action was variable, the reverse also being the case. The greater susceptibility of *Brucella* to penicillin X than to penicillin G may be of practical importance. The consistent inferiority of penicillin K action and the unspectacular action of penicillins F and dihydro F cast doubt on their possibilities as therapeutic agents against gram-negative infections. Penicillin G was consistently the most effective fraction. Combination of penicillin G with streptomycin gave negative results when the concentration of penicillin was less than inhibitory. Only in the case of *P. vulgaris*, which was relatively sensitive to penicillin, was there any effect on the amount of streptomycin necessary for bacteriostasis and on the number of transfers for developing streptomycin resistance. On the other hand, the two antibiotics in combination showed no antagonistic action.

Sodium sulfadiazine added to streptomycin reduced the initial streptomycin requirement two to eight times for four of the test organisms, and the two agents in combination delayed microbial fastness to streptomycin in four out of five instances.

The use of a combination of penicillin, streptomycin, and sulfonamides was first advocated for mixed infections with several species of bacteria. It was also recommended for the treatment of immediately life-endangering infections of uncertain etiology. Successful experiences with combined sulfadiazine streptomycin therapy for brucellosis<sup>3, 11, 12</sup> after failure when each drug was used alone emphasize the possible value of two drugs to control an infection caused by a single bacterial type.

It was noteworthy in these experiments that when penicillin in relatively small amounts was added to the media containing both sulfadiazine and streptomycin, the bacteriostatic concentrations of the latter drugs could be reduced

significantly and retardation in the rate of streptomycin resistance could be effected. There appears to be a penicillin effect not always in evidence when penicillin was used in combination with streptomycin alone. This phenomenon well demonstrates the opportunity for the effective use of relatively small amounts of penicillin against insensitive gram-negative bacteria that is when the organisms can be affected only by marginal or partially inhibitory doses of streptomycin and sulfadiazine.

Complete bacteriostasis of the organisms is essential to eradicate infection. Continued multiplication of bacteria in any combination with streptomycin of these antimicrobial agents in subinhibitory amounts will eventually lead to streptomycin fastness. These studies should therefore not be interpreted to indicate that the management of infections requiring combinations of drugs to control them permits economy in the use of the drugs by the reduction of dosages. On the contrary, maximal therapeutic dosages should be initiated and maintained until the optimal therapeutic response has been achieved.

#### SUMMARY AND CONCLUSIONS

There has been reported in this communication a series of *in vitro* studies concerning the effect of streptomycin combined with penicillin or sulfadiazine or both against gram-negative bacteria with special emphasis on the development of drug resistance.

1 The gram-negative organisms tested show greater variation in sensitivity to penicillin than to streptomycin. The *B. coli* are very sensitive *in vitro* especially to penicillin V. The order of sensitivity for the other genera is as follows: *Aerobacter*, *Escherichia*, *Salmonella*, *Proteus*, *Shigella*, *Pseudomonas*, *Aeromonas*, *Enterobacter*, and *Pseudomonas*. The action of penicillin is bacteriostatic not bactericidal.

2 Streptomycin in combination with penicillin and sulfadiazine is optimally bacteriostatic for gram-negative bacteria and these drugs in combination delay the emergence of drug-fast cultures.

3 These studies suggest that in addition to the use of combinations of penicillin, streptomycin and the sulfonamides for mixed infections caused by several species of bacteria, such combinations may also be useful for infections caused by a single etiological agent. Some clinical evidence to this effect already exists.

4 It is essential when these combinations are employed, that maximal therapeutic doses of the drugs be initiated and be maintained until the optimal therapeutic response has been effected. Economy by reduction of dosage is not indicated and may give rise to drug fastness.

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#### ADDENDUM

These authors show that streptomycin combined with penicillin or with sulfathiazole has an additive or synergistic action in vitro on strains of Brucella, Eberthella, Salmonella, Shigella, and Escherichia

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# I MUMPS VACCINE

## STUDIES ON HUMAN VOLUNTEERS

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THE ever increasing demand of physicians and the American public for additional immunizing vaccines against the common virus diseases has stimulated investigation and development of a new specific vaccine. The work presented herein concerns the virus which causes mumps. Original immunization investigation was carried out with live virus or attenuated cultures as vaccines.<sup>1</sup>

Since the first studies by Johnson and Goodpasture in 1934,<sup>2</sup> and the isolation of a filterable cytotropic virus with a predilection for the parenchymal cells of the pituitary, several attempts have been made to perfect a suitable immunizing product. In March, 1947, an editorial in the Journal of the American Medical Association brought the improved mumps vaccine of Enders and co-workers from the realm of the experimental journals.<sup>3</sup> It placed the possibility of the development of an adequate immunizing vaccine before the practitioners who would have the opportunity to use this vaccine on a large scale.

Recently an entire issue of the Journal of the American Medical Association was devoted to reviews and reports concerning the viruses and the virus diseases, however, little mention was made of any recent advance in active immunization in mumps. Schultz in his discussion of the present status of viruses and virus diseases noted the phenomenal advances and rapidly widening interest in these diseases.<sup>4</sup>

Before any vaccine can be used clinically it must run the usual gamut of experimental trial on human beings. Because of the importance of mumps in military medicine a considerable amount of investigative work was undertaken by members of the United States Public Health Service to work out a practical means of determining susceptibility and of prophylactic immunization. The editorial (J A M A, March 1947) led one to believe that the final product would be a viable vaccine. Should this product, of necessity be a viable virus, its use would be limited by the same factors which limit all viable products. Preparations of the live virus are wholly unsatisfactory since the patient can be given no assurance that he would not become ill with the disease against which immunization is desired.

In a report by Enders and associates in 1946, it was concluded that the mumps virus after twenty-five passages in the chick embryo appeared to be incapable of inducing "typical mumps" in "presumptively susceptible" human beings, when sprayed into the oral cavity. They also found that this material might irregularly lead to the formation of a complement fixing antibody (hereafter designated as CFA).<sup>5</sup>

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Habel has been able to produce parotitis in monkeys on immunization attempts with live egg embryo virus and therefore it seems unlikely that such a viable product would survive clinical criticism. Furthermore, Habel has stated (personal communication) that although he had not used a live virus preparation in human subjects, his virus strains were still able to produce a "good parotitis" in monkeys after thirty egg passages. He stated in unpublished data that he had "good results" with the inactivated virus given intramuscularly to human beings. He concluded from the latter data that there was no good reason for using the active virus.

We propose to present clinical data concerning the use of intracutaneous, nonviable mumps vaccine on a group of unselected human volunteers. The volunteers consisted of medical students, nurses, and patients at the Indiana University Medical Center and Indiana University School of Medicine.

*Mumps Virus Strains*—Two strains of mumps virus were used: one was procured from Dr. K. Habel,\* and the other from Dr. J. Enders†. In tests conducted by us, no differences have been found between the two strains.

*Cultivation of the Virus*—The virus was propagated by inoculating 7-day-old chick embryos which were incubated at 35° C. temperature. Inoculation of the eggs was similar to the usual technique for that of the influenza viruses. The periembryonic fluids were harvested. Tests showed them to contain the highest content of the virus.

*Properties and Control of the Virus*—Since mumps virus propagated by egg passage is not infectious for the smaller laboratory animals, the presence of virus in the egg fluid is determined by complement-fixation (Bengtson technique)<sup>6</sup> and hemagglutination tests (Hirst technique).<sup>7</sup>

Swiss mice were given periodic intranasal instillations of the virus to check chance contamination with other viruses, and homologous and heterologous inhibition of hemagglutination tests were done with mumps and influenza viruses and the corresponding antisera.

*Laboratory Test of Mumps Virus and Production of Mumps Antiviral Serum in Rats and Rabbits*—Each yield of vaccine was tested in five rats, the animals being injected intraperitoneally with 0.2 cc on alternate days, then bled one week after the sixth and final dose of vaccine. With normal rat sera as a control, complement fixation and hemagglutination inhibition tests were conducted with these sera to determine the amount of response. Titers of rats immunized in this way are not unlike those of human beings. Antiviral serum was produced in rabbits by twice weekly intravenous injections of 5.0 cc of the egg-passage antigen. The animals were bled one or two weeks after the eighth and final injection.

*Preparation of Skin Test Antigen*—The periembryonic fluid virus antigen was centrifuged for a short time to clarify it, then diluted to contain 20 complement-fixing antigen units per cubic centimeter. The antigen was then heated to 65° C. for ten minutes, tested for sterility, and Merthiolate (sodium ethyl

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mercuri thiosalicylate, Lilly) 1:10 000 was added as a preservative. The virus is susceptible to the inactivating effects of heat, formalin, ether, and ultra violet irradiation.<sup>8</sup>

Our CF antigen, as present in this skin test antigen, is of course the "virus bound" antigen as described by Henle, Henle, and Harris.<sup>9</sup> It exists primarily in the periembrionic fluids as distinguished from the mumps embryo membrane antigen. According to Henle and co-workers the virus bound antigen is sedimentable at 20 000 revolutions per minute. The corresponding CF antibody, while slower in appearance, persists longer after termination of the disease. On the other hand the "soluble" smaller size nonvirus CF antigen associated with fixed tissue (and with which we have not dealt) gives a short term antibody response in clinical mumps.

#### CLINICAL STUDIES

*Skin Tests*—One tenth cubic centimeter of vaccine containing 20 complement fixing units was placed intracutaneously on the flexor aspect of the forearm.

*Skin Test Reading*—Tests were read as positive if the area of erythema and induration (edema) was larger than 1 cm, negative if the zone of erythema was not greater than 2 cm and showed no evidence of induration. All skin tests were read between twenty-four and thirty-six hours at which time the local reaction was at its maximum.

The color which developed was similar to that seen with the routine Mantoux skin test with tuberculin.

#### SUGGESTED INTERPRETATION (ARBITRARILY SET AFTER OBSERVING 400 SKIN TESTS)

1+	1 cm	—————>	2 cm	diameter
2+	2 cm	—————>	3 cm	diameter
3+	3 cm	—————>	4 cm	diameter
4+	4 cm	—————>	Greater	

*Skin Tests*—Four hundred intracutaneous injections each containing 20 units of complement fixing antibody have been given to date. There has been no evidence of systemic reaction or untoward symptoms. The typical local reaction in persons who had a definite history of mumps and a significant CFA titer was an erythematous indurated area of 2 by 3 cm to 3 by 6 cm. The epidermal tissue acquires hypersensitivity to the antigen following previous experience with the living or killed virus.

Originally the test was designed as a method for demonstration of an allergic response to the heat inactivated virus. It was supposedly a convenient index of determination of susceptibility or resistance of the individual to heat inactivated virus. Evidently from the clinical data furnished by Enders and co-workers in 1945, then inactivated virus produced little or no immunity as measured by the complement fixing procedure. Their antigen was prepared from the infected monkey parotid glands from infected eggs as described.<sup>10</sup>

Hypersensitive state (that is, positive skin test) could first be demonstrated between the second and third month following convalescence.<sup>10</sup> It was therefore

concluded that the skin test would have no value in diagnosis during the active disease. Presumptive diagnosis can be made from the clinical picture and an absolute diagnosis from rising C F A titers and culture of specific virus.

Enders stated that "this skin test material" was weakly antigenic, capable of stimulating the production of antibody in certain of those previously infected but incapable of inducing "de novo" antibody formation in those who have not encountered the virus. Attempts at local passive transfer by technique of Piantz and Kustner could not be demonstrated.<sup>10</sup>

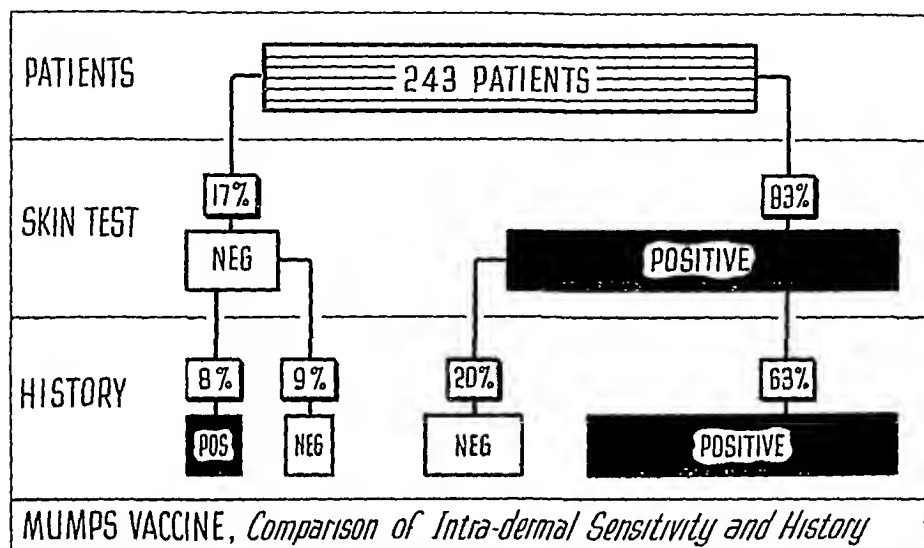


FIG. 1

Human convalescent serum of high titer in C F A was injected into the skin of an individual who had previously failed to react to the skin test material. After an interval of twenty-four hours, 0.1 cc skin test material was injected into the same area. No immediate or delayed reactions were noted.<sup>10</sup>

Enders presented evidence that individuals who were exposed to the virus, yet showed no clinical evidence of the disease, developed a gradually rising C F A titer and rightly concluded that an inapparent infection had been present.

The intracutaneous skin test was applied to a group of volunteers four months after a previous test with the same material. In the majority of the cases there was a more marked reaction to the second dose. Readings which were originally 1+ and 2- were greater than the minimal 4+ (4 cm) reaction in many. A group of volunteers who had negative skin tests originally had 3 to 4+ on their second skin test. Work on C F A titration will be reported later. The opposite arm was used for subsequent skin tests to avoid possibility of local hypersensitivity.

From these results we are forced to question the significance of a second positive skin test in people who have the clinical picture of mumps. We do not believe that a second positive skin test would substantiate a clinical diagnosis.

since we have shown rises in CFA titers comparable to those in mumps following one skin test dose. A rising CFA titer would be very significant if not influenced by inactivated antigen via the intradermal or subcutaneous routes.

Comparative studies of history and intradermal sensitivity on information obtained from individuals having one skin test dose of antigen are seen in Fig 1

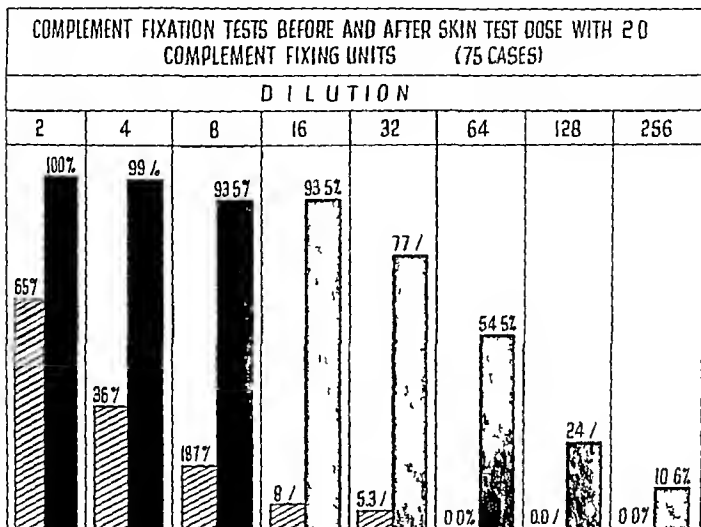


Fig 2

#### CFA STUDIES

Enders (Harvard University) reported complement fixation tests for diagnosis of mumps made from egg antigen to replace the more expensive and less practical source from monkeys.

To show the rise in titer he stated that the test should be made with two specimens of serum, one taken soon after the onset of the disease the second at the end of the third week, for most of the convalescent serums showed a high titer after the second week.

In a series of seventy five unselected cases we gave 20 complement fixing units of heat killed mumps virus vaccine. Prior to intracutaneous injection of the nonviable mumps virus a specimen of blood was obtained to check the complement fixing antibody titer as a control.

Four weeks later a second specimen was collected and its complement fixing antibody titer was also determined. Fig 2 gives us a graphic representation of the CFA antibody titrations before and after the intradermal dose of vaccine.

Enders and co-workers reported positive complement fixation tests on 1+ or 2+ reactions. Our figures are correspondingly much lower since a "positive" C F reaction was read as a positive to the first 2+ reaction, for example

Sample case	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512
	4+	4+	4+	2+	2+	2+	1+	1+	1+
	1 16 was the end point								

It is evident that a marked change took place in the C F antibody titer (Fig. 2)

Studies of specimens taken two weeks following intradermal vaccine showed significant rises in C F antibody titrations above the control serums checked (Results to be reported in a subsequent report)

This is comparable to studies done on convalescing mumps patients by the Harvard group. In four cases described by Enders and co-workers the C F antibody made its appearance before hypersensitivity could be demonstrated. In many cases C F A was present on the first day of clinical symptoms. Dermal hypersensitivity from mumps may last as many as fifty years. This was demonstrated in several elderly subjects.

Comparative studies between history, intradermal sensitivity and complement-fixing antibody titration are illustrated graphically in Fig. 3

#### DISCUSSION AND CONCLUSIONS

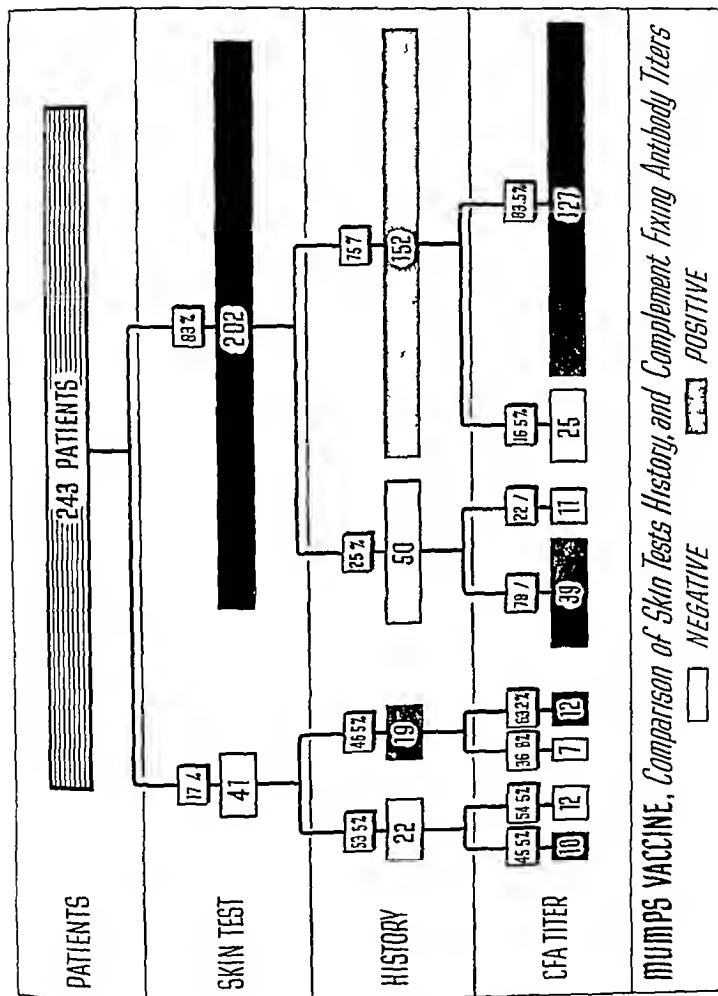
Mumps becomes an important disease whenever proximity of rural and urban people becomes a necessity, for example, military camps, summer camps, and university centers.

It has been reported by Fox that during World War I mumps was third among the diseases causing loss of duty time. Venereal disease and influenza were listed as first and second offenders. The Surgeon General of the United States Army reported that in 1918 mumps accounted for 7.15 per cent of admissions to army hospitals. A total of over 166,000 cases was reported. Wollstein reported epidemic parotitis as the fourth disease in the list of causes of admission to hospitals, but it was the second largest cause for loss of time. In 1940, Surgeon General Parran of the United States Public Health Service stated that mumps was second only to venereal diseases among the disabling acute infections of recruits.

It must be remembered that the incidence, high as it is, is actually relatively higher because most individuals have had the disease in childhood. Radin, in 5,756 cases, found forty-seven with a history of previous attack—0.8 per cent.<sup>11</sup>

In 1943, a "heavily infected" company of 194 soldiers at Camp McCoy, Wis. was studied. Nineteen per cent of the group contracted mumps during the sixteen weeks which elapsed between the onset of the first and last case. This percentage closely parallels our cases who had negative skin tests.

Thirty-two per cent of the soldiers at Camp Wheeler (World War I) had mumps at one time or another during their stay at camp. In the A. E. F. during World War I, one in every twenty white men and one of every six Negroes had an attack.



The total mortality in the U S Army in World War I accredited to mumps as the original diagnosis was 181, but these deaths were associated in every case with a secondary diagnosis which requires consideration in at least partially acquitting mumps of responsibility <sup>12</sup>

Eagle reported a four-year mumps epidemic in one camp in which 2,500 patients were hospitalized. Of 1,664 cases reviewed, epididymo-orchitis occurred in 25.54 per cent <sup>13</sup> (The average age of patients was 24.79 years). In this age group it is a debilitating disease of no small consequence. McGuinness and Gall studied 1,331 cases at Camp McCoy and found 36.2 per cent of the patients with mumps with epididymo orchitis. Orchidotomies were performed on eighty-three soldiers at Camp McCoy <sup>14</sup>

Gregg in 1941 pointed out the high incidence of congenital malformation in children born to mothers who had German measles (rubella) during the early months of pregnancy <sup>15</sup>. Since then, the work has been confirmed by several other workers, more than 400 cases being reported. Incidence of malformations following rubella infection in the first two months of pregnancy is almost 100 per cent. Abnormalities resulting from rubella infection in the pregnant mother: cataract, heart defects, microcephaly and deaf-mutism.

This work stimulated further research to determine the effect of related viruses, for example influenza A and mumps. Hamburger and Habel <sup>16</sup> found that infection of 8-day-old chick embryos resulted in mumps virus multiplication in the allantoic sac, although the mumps virus was lethal for early embryos within five days after infection. They presented well controlled experimental evidence to show that mumps virus has questionable teratogenic effect on early chick embryos (studies have not yet been completed).

Johnson and Goodpasture attempted to confer passive immunity to intra-parotid infection by injection of serum from persons immune to mumps. These attempts failed except in rare cases <sup>17</sup>.

Pooled human serum has been used as a prophylactic measure in individuals exposed to mumps. There are several disadvantages to this method of prophylaxis.

- (1) The period of immunity is probably short.
- (2) Immune serum from convalescent patients should be taken within three weeks of onset date to be most efficient.
- (3) No. 2 makes large scale immunization impractical in epidemics.
- (4) The amount of pooled serum recommended varies between 20 and 40 cc in adults and in our experience has proved very painful to the recipient.
- (5) There is always a danger of transmitting homologous serum hepatitis <sup>18</sup>.

Several considerations present themselves.

- (1) What individuals should be vaccinated?
  - (a) Should the vaccine be used in children?
  - (b) Would the immunity conferred by the disease itself be more lasting than that given by the vaccine? (We are confronted with the problem of the age at which attempts at immunization should begin.)

- (2) Would the vaccine prevent early fetal death and congenital abnormalities due to mumps in infants if pregnant mothers were vaccinated in early pregnancy that is if they give no history of having mumps in the past?
- (3) Does mumps produce a lasting immunity to all manifestations of the disease or merely prevent the recurrence of parotitis?
  - (a) If so should the vaccination be given in spite of a positive history?
  - (b) What place would the vaccine have in immunization of the military forces?
- (4) What place does the vaccine have in a mumps epidemic?
  - (a) Should all nonimmune people (from individual history) who contract cases be vaccinated?
  - (b) Would a vaccination protect them or does the rapid incubation period prohibit development of immunity in time to protect the individual?
- (5) Since immune globulin gives only short passive immunity should both immune globulin and the vaccine be given concomitantly?
- (6) Are "booster" doses necessary? If so when?

Obviously, studies in epidemiology cannot be carried out at a medical center for a scarcity of active cases exists. The ideal proving ground for such immunologic study will rest in large military camps where epidemics are common.

Ratner and Untch in studies of egg vaccine and egg sensitivity concluded that the "Use of vaccines is not dangerous to 99.5 per cent of the general population, which included all the allergic persons who are sensitive to proteins other than egg and about 50 per cent of the persons moderately sensitive to egg."<sup>10</sup>

Demonstration of rising CFA constitutes a valuable clinical diagnostic aid. Central nervous system involvement during mumps had been studied extensively. A rising specific CFA titer for one of the virus infections may help categorize and prognosticate heretofore unclassified encephalitides. Brown and co-workers reported seventy-seven consecutive cases of mumps in which spinal fluid examination was made. Pleocytosis was present in twenty-six (33.7 per cent). Clinical manifestations of meningoencephalitis were recognized in nine cases.<sup>20</sup>

#### SUMMARY

1 Mumps vaccine prepared by inactivation of the living virus (by heat) has been given intracutaneously to a series of human volunteers.

2 Mumps virus strains were propagated on chick embryos. Vaccine was prepared from the heat killed virus.

3 Methods of preparation of the virus and immunization material are presented.

4 Two complement fixing units (0.1 cc of vaccine) were injected intradermally. Hypersensitive reactions seemed to indicate previous contact with the virus in the subclinical or clinical forms. In addition, the same hypersensitivity developed after the first intradermal dose.

5 The mumps vaccine given to our volunteers served as an index of potential susceptibility to mumps and positive reaction implied a previous clinical



or subclinical infection with the virus. The skin test dose caused significant rises in complement-fixing antibody titers and might possibly be a practical method of vaccination. Work is in progress to determine the optimal dose and route of administration.

6. The vaccine caused no untoward reaction in 400 human subjects.

We have no evidence that the immunity which our intradermal vaccine produces will be a permanent one. Follow-up studies of our volunteers will be necessary.

The potential value of the mumps vaccine has, in part, been determined. The actual value of mumps vaccine remains as an unsolved problem.

Long-term immunologic epidemiologic studies must be planned and will be necessary in order to arrive at the solution of the problems presented.

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## SPONTANEOUS AND INDUCED GLOMERULONEPHRITIS IN AN INBRED STRAIN OF MICE

ARTHUR KIRSCHBAUM M.D. PH.D. I. T. BEIL M.D. AND JACK GORDON M.D.  
MINNEAPOLIS, MINN.

A SPONTANEOUS disease simulating chronic glomerulonephritis has not been recognized in animals except in the NH strain of mice<sup>1</sup> with which this report deals. This stool of mice is of special significance because it represents a population in which the incidence of the disease is high. It should be possible therefore to study etiologic factors under controlled conditions.

The NH stock was developed by Dr. L. C. Strong, of the Yale University School of Medicine. In the fourth inbred generation four sublines were separated. One of these was established in Minnesota in the eighth inbred generation in 1941. Although the present stool of the Minnesota subline is represented by descendants of a pair of mice brought to Minnesota at that time, brother-sister mating was not carried out until 1943 when it was recognized that the animals develop glomerulonephritis. Subsequently selection of mice possessing genetic susceptibility toward the disease has been attempted by mating the progeny of animals which develop nephritis.

The NH mice are very obese animals at the age of six months. Old adults may attain a weight of 50 grams which is almost twice that of the average inbred mouse. Subcutaneously and intra-abdominally there are very large deposits of fat. The females especially, but males as well, develop tumors of the adrenal cortex when the animals are over 1 year of age. Leucemia appears spontaneously in about 5 per cent of the mice, also occasional lung tumors. The males develop a distention of the bladder spontaneously at about 1 year of age in association with which hydronephrosis appears. The average life expectancy has been approximately 600 days.

Initially the animals were maintained in the laboratory for no particular purpose except to provide mice for routine experiments that necessitated no special type of genetic susceptibility. It was noted that many NH females which died between the ages of 11 and 20 months were extremely edematous at autopsy. There was often in excess of 1 cc of clear fluid in the chest and more than 2 cc in the peritoneal cavity. The subcutaneous edema was so extreme that the distance between the abdominal muscles and epidermis was expanded to a half inch. This fluid was usually serous and colorless although subcutaneously it was often yellow tinged. Animals which developed ascites, hydrothorax, andanasæra usually died within a week. In certain instances this edematous condition subsided temporarily but there was always recurrence with death in every case. No animal survived more than three weeks after the

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Initially the animals were maintained in the laboratory for no particular purpose except to provide mice for routine experiments that necessitated no special type of genetic susceptibility. It was noted that many NIH females which died between the ages of 11 and 20 months were extremely edematous at autopsy. There was often an excess of 1 cc. of clear fluid in the chest and more than 2 cc. in the peritoneal cavity. The subcutaneous edema was so extreme that the distance between the abdominal muscles and epidermis was extended to a half inch. This fluid was usually serous and colorless although subcutaneously it was often yellow tinged. Animals which developed ascites, hydrothorax, andanasica usually died within a week. In certain instances this edematous condition subsided temporarily but there was always recurrence with death in every case. No animal survived more than three weeks after the

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onset of the clinical disease. There appeared to be no antecedent occurrence of disease, renal or otherwise. Animals which apparently had been in good health suddenly became quite edematous and died. The disease has occurred exclusively in females.

Grossly the kidneys were of normal size, but always pale. Examination of the kidneys microscopically revealed that the seat of the pathologic disturbance was in the glomerulus. The glomeruli exhibited changes which correspond closely to those of human chronic glomerulonephritis, and the tubules contained protein casts. Microscopically the condition is quite different from the amyloid (cystic) disease which occurs in other stocks of mice, especially the Strong A<sup>3</sup>. A glomerular lesion similar to that found in the NH mice also has been seen by the authors in two mice of the F strain (Fig. 6, 13 and 14).

The following discussion presents observations on the spontaneous disease in this stock of animals, and a similar condition induced by the administration of urethane.

#### MATERIALS AND METHODS

A control group of NH mice was maintained to determine the incidence of spontaneous chronic glomerulonephritis. All animals were bred and fed a diet of Purina Fox Chow with no supplement. Water was always available. Often the disease was diagnosed subsequent to the death of the animal since the mice died very soon after the clinical onset of the disease. Complete autopsies were done, sections being cut at 5 micra and stained with hematoxylin and eosin. In addition all kidneys were stained with azocarmine after fixation in Zenker's fluid. Certain kidneys were fixed in formalin and stained for amyloid.

After the onset of edema the mice were placed in specially constructed glass cages for collection of urine. Albuminuria was ascertained by the routine method of boiling and acidification. For blood determinations blood was drawn immediately after the animals had been killed with ether anesthesia. Blood was taken from the vena cava and coagulated. Plasma and blood obtained in this way were used for determining blood urea nitrogen, nonprotein nitrogen, plasma proteins, hemoglobin, red blood cell counts, and the titer of antibodies against streptococci. The Karr method<sup>4</sup> was used for the determination of blood urea nitrogen, the method of Koch and McMeekin<sup>5</sup> for nonprotein nitrogen and plasma protein and Bloor's method<sup>6</sup> for cholesterol.

Agglutinin titers were determined against strains of *Streptococcus viridans* and *Streptococcus hemolyticus* obtained from human sources. Twenty-four hour broth cultures were used.

Pure cultures of *Str. viridans* were obtained from the pharynges of both normal and nephritic strain NH mice. These organisms were autoagglutinating and could not be used for agglutination tests. However, when injected intraperitoneally into mice, the antiserum thus produced contained antibodies active against the streptococci from the human source. This procedure was carried out to demonstrate the common antigenic properties of streptococci from mouse and human sources, and thus justify the use for agglutination studies of the *Str. viridans* and *Str. hemolyticus* of the human pharynx.

To determine whether the plasma of nephritic mice was nephrotoxic and contained antibodies specific for mouse renal antigen, the plasma was tested using the method of Cavelti and Cavelti<sup>7</sup> for the preparation of the renal antigen. Perfused kidneys of both normal and nephritic mice were used as the source of antigen. One part perfused kidney to 20 parts saline was used in making the test antigen for determining agglutination titers. Control studies were carried out on nephrotoxic rabbit plasma obtained from rabbits injected periodically with a saline extract of perfused mouse kidney.

## INCIDENCE OF SPONTANEOUS GLOMERULONEPHRITIS IN FEMALE NH MICE

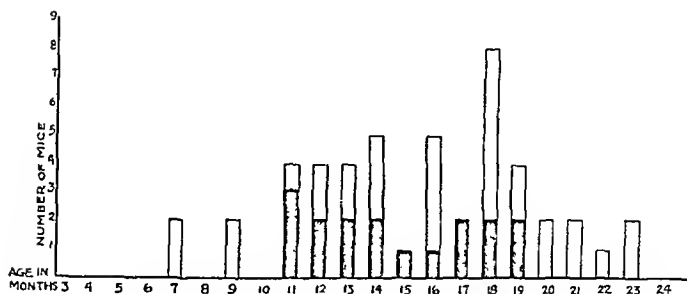


Fig 1—Illustrates the age incidence of spontaneous glomerulonephritis in female mice of the NH strain

## INCIDENCE OF SPONTANEOUS GLOMERULONEPHRITIS IN MALE NH MICE

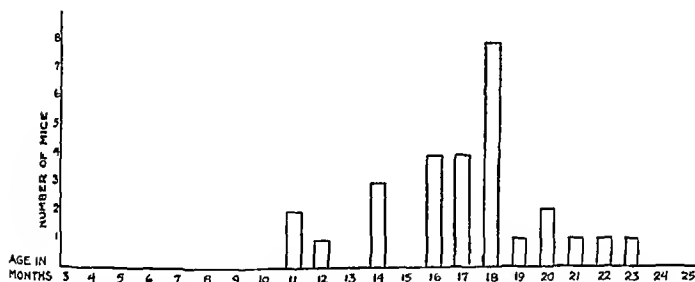


Fig 2—Illustrates the absence of glomerulonephritis in male mice of the NH strain. The males were of comparable age to the group of females in which the incidence of glomerulonephritis was high

## INCIDENCE OF GLOMERULONEPHRITIS IN CASTRATED MALE NH MICE

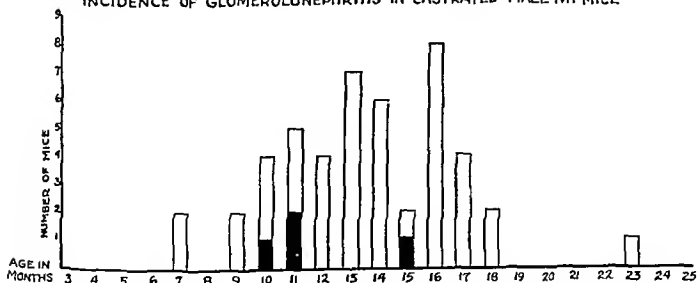


Fig 3—Illustrates that a small per cent of castrated males developed glomerulonephritis

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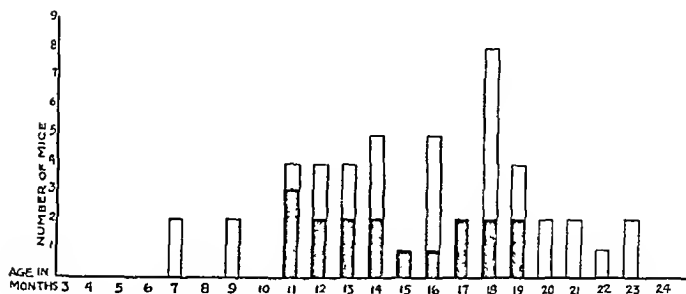


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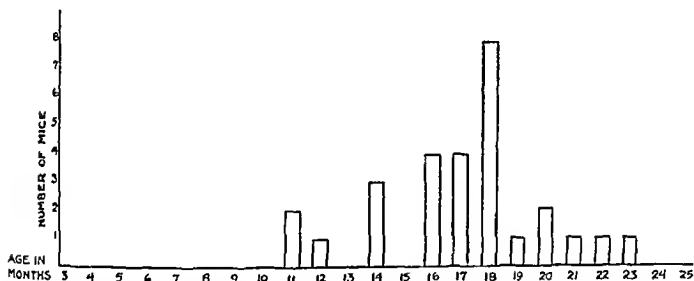


Fig 2—Illustrates the absence of glomerulonephritis in male mice of the NH strain. The males were of comparable age to the group of females in which the incidence of glomerulonephritis was high

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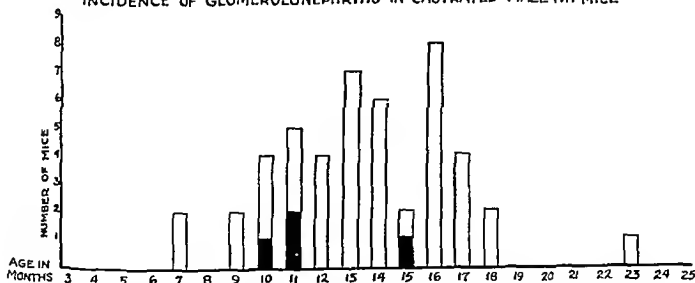


Fig 3—Illustrates that a small per cent of castrated males developed glomerulonephritis



## INCIDENCE OF URETHANE INDUCED GLOMERULONEPHRITIS

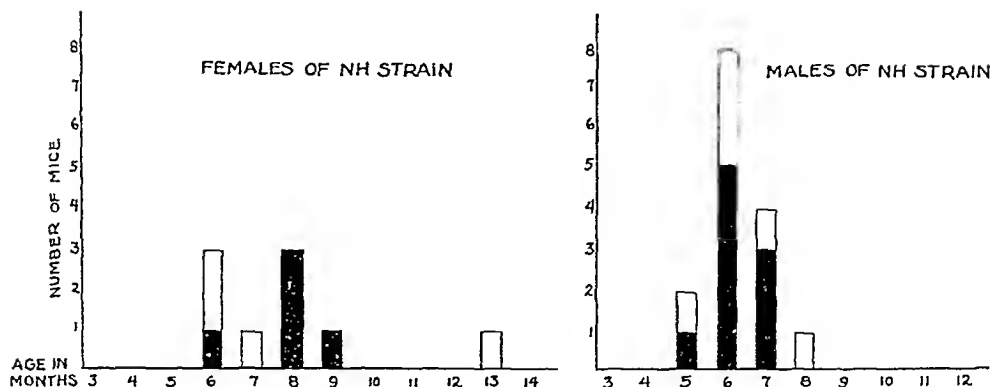


Fig 4—Illustrates the age of occurrence and incidence of urethane-induced glomerulonephritis in NH mice of both sexes

The age incidence of spontaneous nephritis in female NH mice is presented in Fig 1. Seventeen out of forty-eight females developed the disease, which appeared between the ages of 11 and 19 months. By contrast, none of twenty-eight males, all of which lived eleven or more months, developed nephritis (Fig 2). Four of forty-seven castrated males developed the disease (Fig 3). These observations were on pedigreed stock. Previous to inbreeding, twenty-four cases had been observed in a group of fifty-nine females, and one questionable case in seventy-one males.

All female mice 11 months or more of age had estrogen-secreting adenomas of the adrenal cortex, whereas such adenomas did not appear spontaneously in males. When either males or females of this stock are castrated, these adenomas are present within five months. Thus, all the castrated males possessed cortical adenomas which secreted estrogenic hormone.<sup>10</sup>

Although the disease did not appear spontaneously in males, animals of both sexes injected with urethane in the manner described developed clinical nephritis (Fig 4) which was grossly indistinguishable from the spontaneous disease. Neither Bagg albino, CBA, Strong A, nor FA mice responded in a similar manner. Thus, in only mice of the "high nephritis" NH stock was glomerulonephritis induced by urethane.

Fig 5—5. Renal corpuscle of adult male NH mouse. Parietal layer of Bowman's capsule composed of cuboidal epithelial cells indicating androgenic stimulation. Capillary loops of glomerulus well shown. Delafield's hematoxylin-base fuchsin. Photomicrograph X350.

6. Renal cortex from a spontaneous case of chronic glomerulonephritis in an NH female mouse. The glomerular capillaries are almost completely occluded and some hyaline changes are noted. Azocarmine stain. Photomicrograph X200.

7. Glomerulus from a spontaneous case of chronic glomerulonephritis in an NH female mouse. The glomerular capillaries are completely occluded by endothelial cells. This lesion corresponds closely to human acute glomerulonephritis. Azocarmine stain. Photomicrograph X275.

8. Glomerulus from spontaneous chronic glomerulonephritis in an NH female mouse. The central capillary basement membranes have thickened and fused to form hyaline masses in the centers of the lobules. Many of the capillaries in the periphery of the lobules are still open. This closely resembles the early chronic stage of human glomerulonephritis. Azocarmine stain. Photomicrograph X350.

9. Kidney section from spontaneous chronic glomerulonephritis in an NH female mouse showing casts in the tubules. Azocarmine stain. Photomicrograph X150.

10. Amyloid deposits in the renal corpuscles of an NH female mouse following long term administration of urea. Note that this lesion is entirely different from glomerulonephritis. Hematoxylin eosin. Photomicrograph X150.

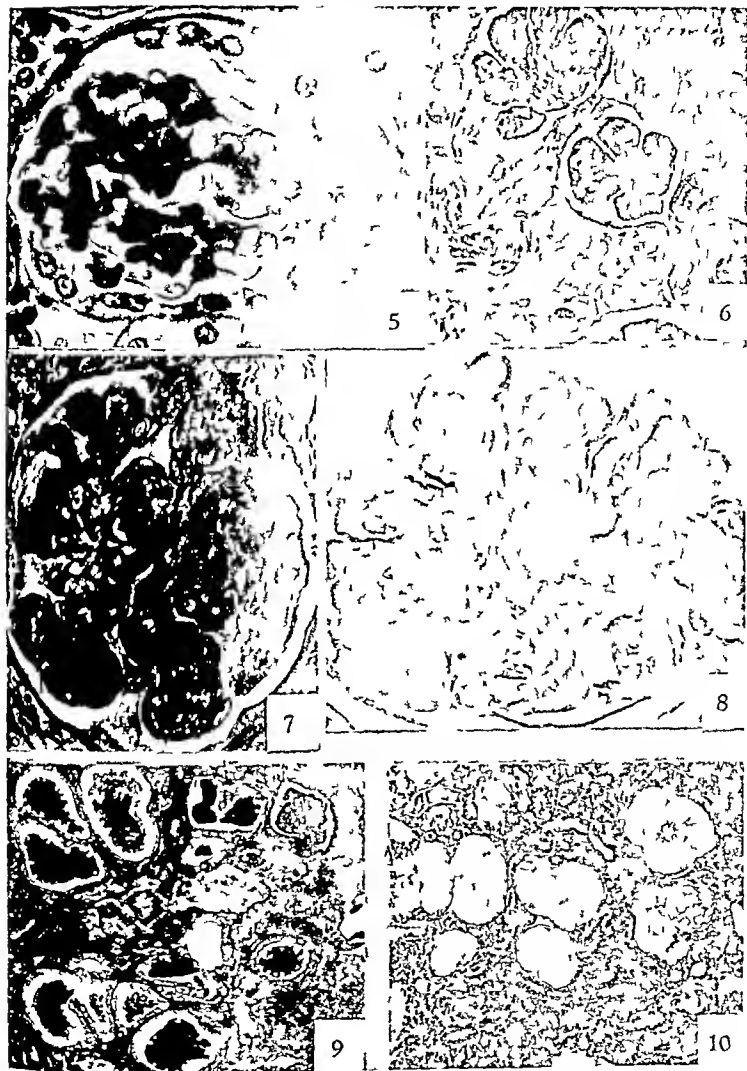


Fig 5—(Legend on opposite page)

In urethane-treated mice the glomerular lesions were not identical with those seen spontaneously (Fig 6, 11, and 12). In the latter the endothelial proliferation is the most prominent pathologic feature. In urethane-induced nephritis, thickening of the capillary basement membrane is much more conspicuous than endothelial proliferation (Fig 6, 11). The end result is a fibrotic glomerulus (Fig 6, 12) which is smaller than the hyaline glomerulus of the spontaneous case (compare Fig 5, 8 and Fig 6, 12). The development of this type of glomerulus is determined not only by urethane but also the genetic constitution of the host. Although the glomeruli of neither Bagg albino nor CBA mice became fibrotic as a result of the administration of urethane, many of the hybrids (receiving urethane) between either of these stocks and NH mice developed the small hyaline glomeruli. The tendency toward *clinical* nephritis is not as great in hybrids as in pure NH mice.

Lesions other than those in the kidneys were noted in mice injected with urethane. Adenomas of the lung<sup>11</sup> appeared in all stocks of mice thus treated. Cavernous hemangiomas were present in the livers of certain animals receiving urethane, especially mice of the Bagg albino strain and hybrids between this and other strains.

#### DISCUSSION

The glomerular alterations occurring spontaneously in female NH mice seem to be identical with those of human chronic glomerulonephritis.<sup>12</sup> Secondary tubular atrophy is not prominent, however, in the mouse kidneys. Perhaps an insufficient period of time elapses in the mouse between glomerular closure and death so that tubular atrophy may result. Glomerular lesions were widespread (Fig 5, 6) and glomerular closure seemed to be rather complete in some cases.

Determination of antibodies failed to reveal any information indicating an association of infection with the mouse disease. Some of the glomeruli exhibited endothelial proliferation (Fig 5, 7) as in human acute glomerulonephritis associated with bacterial infection. It is possible that the human and mouse diseases are pathologically and clinically similar, but etiologically unrelated. Or perhaps chronic glomerulonephritis is not necessarily associated with antecedent bacterial (streptococcal) infection.\* High antistreptolysin titers have been found in human acute but not chronic glomerulonephritis.<sup>13</sup>

Mouse and rat glomeruli exhibit a sexual dimorphism<sup>14</sup> (Fig 5, 5), and there is a sex difference in the occurrence of this renal glomerular disease. That this susceptibility to the development of glomerulonephritis is, at least to some extent, on a hormonal and not only a genetic basis is illustrated by the occurrence of the disease in castrated males with estrogen-secreting adenomas of the adrenal cortex. The incidence of the disease in castrates is admittedly small. The possible inhibiting effect of androgen on the development of spontaneous and induced nephritis should be tested.

\*Dr. B. J. Clawson of the Department of Pathology, University of Minnesota, has informed us that he has found high titers of streptococcal (*Str. viridans* and *Str. hemolyticus*) agglutinins in several cases of human chronic glomerulonephritis.



Fig 6—11 Thickening of glomerular capillary basement membrane in urethane treated NH mouse. This lesion bears some resemblance to human lipoid nephrosis. Azocarmine stain. Photomicrograph X350.

12. Small fibrotic glomerulus resulting from urethane treatment. The capillary lumens are occluded by the thickened basement membranes. Such glomeruli were found only in urethane treated mice of the NH strain. Azocarmine stain. Photomicrograph X300.

13. Glomerulus from a strain F mouse with chronic glomerulonephritis. The disease occurs occasionally in this strain and the lesions are similar to those found in NH mice. Azocarmine stain. Photomicrograph X300.

14. Glomerulus from strain F mouse showing one portion with capillaries plugged as a result of endothelial proliferation. Note mitotic figure. Hematoxylin eosin. Photomicrograph X350.

Blood studies indicate a high degree of renal insufficiency and anemia in the advanced disease. These animals represent good material for the investigation of the anemia associated with uremia. Hypercholesterolemia is, perhaps, to be related to the low level of plasma proteins.

The concomitant occurrence of spontaneous tumors of the adrenal cortex and renal disease in females of this stock may be merely coincidental. Animals injected with urethane developed glomerulonephritis in the absence of adrenal cortical hyperplasia. There is no evidence that these adenomas may secrete hormones other than the sex steroids.

Since other investigators have been able to induce glomerulonephritis in rats by the injection of nephrotoxic sera,<sup>15</sup> determination of antibodies versus renal antigen was made. There was nothing to indicate that specific antibodies against a renal antigen were present in the plasma of nephritic mice (Table VI).

TABLE VI AGGUTININ TITERS AGAINST NORMAL AND NEPHRITIC RENAL ANTIGEN IN THE PLASMA OF NORMAL AND NEPHRITIC MICE

TOTAL NUMBER OF MICE	TREATMENT	AGGUTINATION TITER VS		NUMBER OF MICE
		NORMAL KIDNEY	NEPHRITIC KIDNEY	
7 Nephritic (spontaneous)	None	Neg 1 50 1 100 1 200		4 1 1 1
2 Nephritic (spontaneous)	None		Neg 1 100	1 1
8 Normal	None	Neg 1 50 1 100		6 1 1
2 Normal	None		Neg	2
4 Normal	Kidney inject	Neg 1 200		2 2
5 Normal	Kidney strep inject	Neg 1 100 1 200		2 1 2
4 Normal	Liver strep inject	Neg 1 400		3 1

0.1 cc nephritic rabbit sera (anthrax kidney) with titers of 1 800 or more induced passive agglutination in mice. The values obtained in this table may represent nonspecific agglutination.

The influence of streptococcal infection and streptococcal toxins on the development of renal glomerular lesions in NH mice should be determined. Preliminary experiments indicate that inoculations of *Str. viridans* over a period of several months do not cause nephritis to appear precociously in NH mice. It should also be determined whether substances related to urethane (ethyl carbamate) affect the renal glomeruli. Nephritis was not induced by injections of either urea or thiourea although amyloid disease appeared in some animals so treated (Fig 5, 10). Weekly anesthetic doses of Sodium Amytal or magnesium sulfate also failed to induce renal disease in NH mice. Thus, anesthesia per se (urethane is a general anesthetic) would appear not to be a factor of importance in the response of NH mice to urethane.

The genetic aspects of NH nephritis have not been investigated although the disease has been observed by the authors in  $F_1$  hybrids between the NH and

other strains of mice. This is in contrast to the amyloid cystic disease of strain A mice, susceptibility to which is inherited as a Mendelian recessive.<sup>16, 17</sup> Amyloid cystic disease also appears in mice of both sexes. Histologic studies show clearly that the occlusion of the NII glomeruli is not the result of deposition of amyloid. Spontaneous amyloid disease does occur occasionally, however, in mice of the NII stock.

The tendency to develop fibrotic glomeruli following the administration of urethane is also not heritable as a simple Mendelian recessive. All types of hybrids ( $F_1$ ,  $F_2$  and back crosses) developed urethane glomeruli following twenty weekly doses, although chronic nephritis appeared only occasionally in hybrids. It is unlikely that the susceptibility of NII mice to both spontaneous and urethane-induced nephritis is without causal relation. Although histopathologically the spontaneous and urethane-induced diseases are not identical, in both instances there is occlusion of the glomerular capillaries and the renal lesions are confined to the glomeruli.

Preliminary observations suggest a rise in blood pressure in mice with glomerulonephritis; this subject will be considered in a future communication. Histologically the amount of renal glomerular closure may be extreme. However, the small amount of tubular degeneration suggests that renal ischemia is not general. Perhaps the renal tubules of mice are supplied from an arterial source other than the efferent arterioles.

#### SUMMARY

1. A disease resembling human chronic glomerulonephritis occurs spontaneously in female mice of the NII stock. This disease is characterized by albuminuria, edema, nitrogen retention, hypoproteinemia, anemia, and hypercholesterolemia. The glomerular lesions are histologically identical with those of human chronic glomerulonephritis, but tubular atrophy is inconspicuous and the kidneys do not become contracted. Blood pressure is perhaps moderately elevated.

2. The disease appears in about 40 per cent of intact females and 10 per cent of castrated males; nephritis does not occur spontaneously in males. The age of occurrence is from 11 to 20 months.

3. The titer of antibodies specific for streptococci in the blood of nephritic mice is the same as in nonnephritic mice of the NII and other strains.

4. A disease similar to but not pathologically identical with the spontaneous nephritis was induced in strain NII mice of both sexes by the administration of urethane. Four other inbred stocks resisted the induction of glomerulonephritis by this drug.

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# STREPTOCOCCUS VIRIDANS ENDARTERITIS OF AN ARTERIOVENOUS ANEURYSM

CURED BY PENICILLIN AND SURGICAL EXCISION

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MASSIVE doses of penicillin with the aid of secretory blocking agents such as Cytomamide, have brought the formerly fatal subacute bacterial endocarditis within range of therapeutic control. However, even after cure of the infective agent, cardiac limitation due to valvular pathology remains and the danger of recurrence of the same illness is always present. This is also true in instances of bacterial endocarditis with the vegetations occurring on congenital cardiac anomalies. In subacute bacterial endarteritis in which the infected vegetations occur on arteriovenous communications outside the heart, it is possible to effect a permanent cure with penicillin and surgical removal of the aneurysm. Such arteriovenous communications may be congenital as in patent ductus arteriosus or may be the result of trauma as in arteriovenous aneurysms.

A few cases of bacteremia associated with arteriovenous aneurysms have been reported.<sup>1</sup> The first cure of bacterial endarteritis in such an aneurysm was recorded by Hamman and Runkhoff<sup>2</sup> in 1935. In this case the arteriovenous aneurysm of the external iliac artery and vein resulted from a gunshot wound inflicted nine years previously. Surgical removal of the aneurysm was the only treatment in this case. In 1939 Leaman<sup>3</sup> reported the case of an arteriovenous aneurysm in a patient with bacteremia. Following surgical removal of the aneurysm the patient continued to have fever and apparently still had bacterial endocarditis. In 1942 Touff<sup>4</sup> Lande and Kroop reported a surgical cure of an infected arteriovenous aneurysm with resultant clearing of septikemia. The next recorded cure was by Lipton and Miller<sup>5</sup> in 1944. Septikemia associated with an arteriovenous aneurysm of the femoral vessels was cured by excision of the aneurysm. In 1946 Shumacker, Welford and Carter<sup>7</sup> reported the case of a 26 year old soldier who developed bacterial endarteritis in an arteriovenous aneurysm caused by a grenade wound two years before development of septikemia. This patient was treated with penicillin in doses of 100,000 to 200,000 units daily, with subsequent negative blood cultures. Surgical removal of the aneurysm effected a cure although viable organisms were present in the vegetations at the time of operation.

The case of bacterial endarteritis in an arteriovenous aneurysm described in this report was cured by removal of the aneurysm. The patient was first treated intensively with penicillin which resulted in sterilization of the blood stream before operation.

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CASE REPORT—H H, an 18 year old white man, was admitted to the University of Kansas Medical Center on Jan 6, 1948. His chief complaints were fatigue, shortness of breath, palpitation of the heart, fever, and profuse sweats. There was no history of past disease which might have contributed to his present illness.

On Aug 2, 1944, this patient received an accidental gunshot wound of the right thigh while hunting. The femoral artery and vein were severed about 6 cm below the inguinal ligament. This resulted in gangrene of the foot and an amputation was done at the juncture

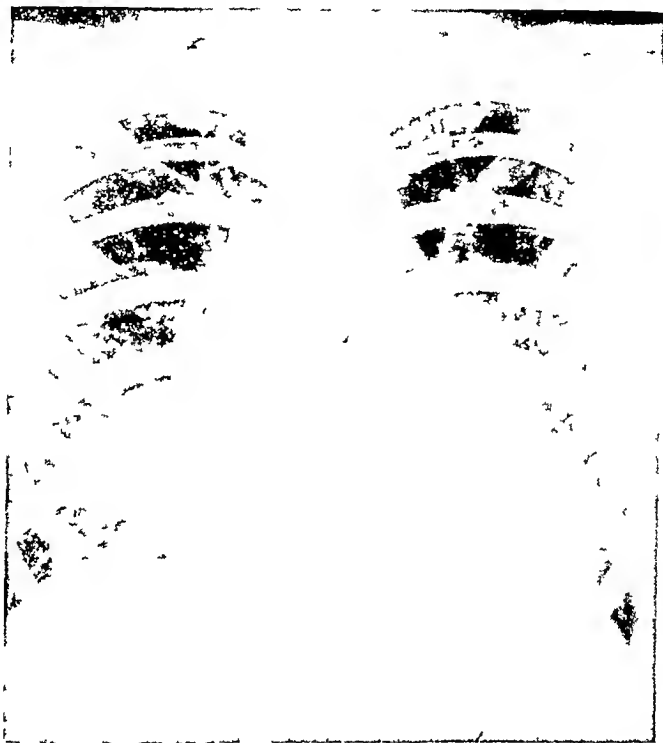


Fig 1—Roentgen ray picture of the heart before operation

of the middle and upper thirds of the right leg. Recovery was prompt and an artificial limb was fitted within six months after the amputation. Several months after the accident the patient noticed that he had a throbbing area in the upper right thigh, but since it did not cause pain or inconvenience he was not concerned about it.

He gave a history of frequent toothache, sore throat, and upper respiratory tract infections during the past two or three years. Eighteen months prior to admission to the hospital he began to have shortness of breath on exertion, weakness and fatigue, associated with "pounding" of the heart. These symptoms grew progressively worse until six months before entering the hospital, when the patient developed chills, fever, nausea, and vomiting. He also had frequent throbbing frontal headaches. He was treated by local physicians who noted that he had an enlarged heart, cardiac murmur, daily fever, and anemia. One of his physicians made a blood culture which was reported to contain an abundant growth of long chain streptococcus, and a diagnosis of endocarditis was made.

When this patient entered the hospital he was poorly nourished and obviously acutely ill. His temperature was 100.2°. The pulse was regular and of the water hammer type. De Musset's sign was present. The blood pressure in the right arm was 130/30 and in the left



Fig 2—Photograph of excised aneurysm (Pointer indicates vegetation)

arm 130/20 The entire precordium throbbed and pulsated, and the heart was enlarged as determined by percussion. There was a loud rasping systolic murmur and a soft diastolic murmur heard in the mitral area. Harsh systolic murmurs were heard to the left of the sternum in the second and third interspaces over the aortic area and over the carotid vessels. All peripheral vessels were throbbing, and showed the typical Corrigan type of pulse. The spleen was enlarged. An ovoid mass 4 by 6 cm in size was palpated in the right thigh just below the inguinal ligament. This mass was pulsating and expansile and a to and fro machinery type murmur was heard over the mass. The murmur was transmitted to the right lower quadrant of the abdomen and along the thigh to the knee. When the pulsation was obliterated by pressure over the femoral vessels, the pulse rate was reduced from 100 to 70. No temperature changes were noted in the right lower extremity. The leg had been amputated below the knee.

Examination of the urine showed a faint trace of albumin. The hemoglobin was 8.4 Gm and the white cell count was normal. The Wassermann and Kahn tests were normal. The total proteins were 6.04 Gm with normal albumin globulin ratio. The blood sedimentation showed a fall of 32 mm. at the end of an hour. Blood cultures on the second and third days after admission showed growths of *Streptococcus mitis*. With sensitivity tests to penicillin and sulfadiazine, the growth was inhibited by 0.48 unit per cubic centimeter and 25 mg per cubic centimeter respectively.

By x-ray examination the heart was enlarged, measuring 18 cm in its greatest diameter (Fig 1). One half of the inside diameter of the chest measured 15 centimeters. Electrocardiogram readings were: heart rate 88, P-R interval 0.16, rhythm sinus, QRS complexes upright in the limb leads, small S2, T waves upright in all leads, sound tracings of the heart showed diastolic and systolic murmurs at the apex associated with an increased first sound and evidence of a systolic murmur in the pulmonic area.

On the sixth day after admission to the hospital, penicillin in 300,000 unit doses was started and continued every three hours by intramuscular injections. Cironamide was also given in 2 Gm doses every three hours. Within twenty-four hours the temperature was normal and remained normal thereafter. Penicillin levels varied between 8,170 and 17,306 units per cubic centimeter. After three days of penicillin therapy the blood culture was negative and remained negative. Two transfusions were given which increased the hemoglobin to 11.7 grams. Although palpitation and fatigue continued, the patient was otherwise symptom free and had gained weight during thirty days of treatment. He received a total of 72,000,000 units of penicillin over a period of thirty days. The sedimentation rate showed very little change.

Forty-five days after admission to the hospital and thirty-nine days after the beginning of penicillin treatment, the arteriovenous aneurysm was completely excised. During the operation, as soon as the vessels leading to and from the aneurysm were clamped or ligated the pulse quickly dropped from 105 to 70 and the blood pressure increased from 130/30 to 140/90. One hour after operation a reduction in size of the heart could be detected by physical examination, as verified by several of the staff. There was a faint presystolic murmur in the mitral area but no systolic murmur was heard. No thrills were detected. Faint systolic murmurs were heard in the tricuspid, aortic and pulmonary valvular areas. On the day after operation the heart appeared to be relatively normal in size, the heart sounds were of good quality, and no thrills or murmurs were detected. The blood pressure averaged 140 systolic and 70 to 90 diastolic.

The excised aneurysm measured approximately 5.5 by 4 by 3 cm (Fig 2). The cavity of the aneurysm had numerous sacculations. Several pinkish and grayish white papillary projections were formed outside the sac. The pathologist reported a diagnosis of aneurysm with arteriosclerosis, calcification, ulceration, and acute and chronic inflammatory reaction with vegetations. Shadowy granular forms were found in the vegetations which suggested chains of bacteria.

The postoperative course was uneventful. The wound healed per primam. Repeated blood cultures were negative. The blood count and sedimentation rate returned to normal. Three days after operation the transverse diameter of the heart measured on the x-ray film was 16.5 cm, and ten days postoperatively the heart measured 14.8 cm in diameter (Fig 3). The heart appeared to be normal in size. The electrocardiogram was normal. No heart murmurs were detected with repeated sound tracings.

When dismissed from the hospital on March 11, 1948, approximately two months after admission, the patient apparently had recovered completely.

#### DISCUSSION

This 18-year-old patient on physical examination presented findings which were indicative of endocardial disease of the valves of the heart. In addition to marked enlargement of all chambers of the heart, he showed harsh murmurs

over the precordium, both systolic and diastolic in time. He gave, however, no history of previous cardiac difficulties, no evidence of congenital heart lesions early in life, nor did he present any findings of peripheral embolic phenomena. That no valvular lesions existed was proved by the rapid return of his heart to normal size in ten days and the subsidence of all murmurs within twenty-four hours following operation.

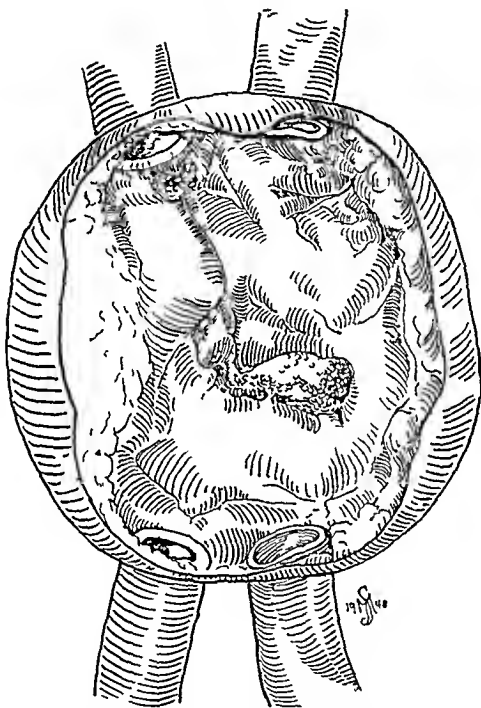


Fig. 3.—Schematic drawing of excised aneurysm.

It is a well known fact that transient streptococcus bacteremia occurs frequently in respiratory infections, sinus infections, and in dental extractions. As in the case with damaged valvular endocardium, the damaged endothelium of an arteriovenous aneurysm will serve as a focus upon which bacteremia may settle and multiply. The infected aneurysm will then serve as a constant source of bacteremia. This patient was started on large doses of penicillin before sensitivity tests of his organism had been carried out and it



Fig 4—Roentgen ray film of the heart four days after operation



Fig 5—Roentgen ray film of the heart ten days after operation

was not deemed wise to reduce the dosage when once started. Vesell and Kross<sup>8</sup> recommend that bacterial endarteritis engrafted upon a patent ductus arteriosus should be treated vigorously with penicillin before operation. Should small emboli be dislodged during the trauma of surgery, morbidity is certainly reduced by earlier sterilization of vegetations. That this patient's vegetations were sterile was verified by culture and by microscopic study which revealed only shadowy forms of pre-existent organisms.

The fact that no vascular insufficiency evidenced by edema or temperature changes developed in the extremity following operation is not surprising since Pratt<sup>9</sup> reports that three to six months is sufficient time for completely adequate collateral circulation to develop in the presence of an arteriovenous aneurysm.

The recognition of endarteritis in arteriovenous aneurysms is particularly important at this time since an increase in frequency of such infections may be anticipated in the future as a result of wounds of blood vessels during the last war. Certainly it is of importance to bear in mind such a possibility in all cases of streptococcus septicemia since a dramatic and permanent cure can be effected in such cases.

#### SUMMARY

A fifth case of streptococcus septicemia due to endarteritis of an arteriovenous aneurysm is presented. A cure was achieved by the use of penicillin and surgical removal of the aneurysmal sac.

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# THE RESISTANCE OF RECENTLY HEALED EXCISIONAL ULCER OF THE STOMACH TO HISTAMINE-INDUCED ULCER

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## INTRODUCTION

IT HAS long been known and repeatedly confirmed that a surgically produced defect in the gastric mucosa will heal spontaneously within three weeks, even in the presence of normal gastric secretion of acid and pepsin. Dragstedt<sup>1</sup> made acute ulcers from 1.0 to 1.5 cm in diameter in the mucosa of a Pavlov pouch, healing occurred in five to fifteen days. Matthews and Dragstedt<sup>2</sup> found that ulcers made in the pyloric mucosa of dogs with silver nitrate healed in fifteen to eighteen days. Morison<sup>3</sup> observed in the dog that defects 2 cm in diameter in the fundic and pyloric mucosa healed in two weeks. McIlroy<sup>4</sup> found that ulcers of the stomach, produced by burning with a hot wire, would heal in fourteen days in the fundus and seventeen days in the pylorus. Bolton<sup>5</sup> observed that experimental ulcers healed within three weeks regardless of their location in the stomach.

It also has been demonstrated that an excisional ulcer of the stomach will be delayed or prevented from healing if histamine in sufficient dosage is given within one to two days postoperatively. McIlroy<sup>4</sup> produced acute ulcers in the pylorus of seven cats and injected 10 to 20 mg of histamine acid phosphate subcutaneously on alternate days. In eight control cats the pyloric lesion required seventeen days to heal. In the cats given histamine, the initial lesion, instead of healing in from eight to fifteen days, had extended in six of the seven animals and perforated in one. Flood and Howes,<sup>6</sup> using cats and dogs, interpreted their observations as indicating that the injection of 1 to 2 mg of histamine per kilogram body weight twice daily interfered with the healing of prepyloric mucosal defects but not consistently with defects in the mucosa of the cardia. Similar results were reported by O'Shaughnessy<sup>7</sup> and Carnot and co-workers.<sup>8</sup> Price<sup>9</sup> has recently shown that the daily administration of histamine in beeswax to dogs causes extension of surgical excision ulcers in the stomach. The apparently negative results obtained by Friedenwald, Feldman, and Morrison<sup>10</sup> may be attributed to the low dose of histamine that was used (average daily dose of 0.8 to 1.2 mg given in one to three doses subcutaneously).

Hay, Vaico, Code, and Wangenstein<sup>11</sup> have shown that superficial and perforating ulcers of the pylorus and duodenum could be produced in dogs by the daily administration of 30 mg of histamine (base) in mineral oil and beeswax medium.

It was considered of interest to study the sensitivity of the healed excisional ulcer to histamine-induced ulcer.

## METHODS

Thirty three mongrel dogs ranging in body weight from 7 to 15 kg, were used. Surgical excisional ulcers were made on the lesser curvature of either the fundic or pyloric portion of the gastric mucosa. The pyloric defects were made 2 to 4 cm from the pyloric sphincter. Under ether anesthesia an appropriate longitudinal incision was made in the anterior wall of the stomach and the mucosa of the lesser curvature was everted through the wound. Approximately 1 sq cm of gastric mucosa was excised from the submucosa with scissors. After closure of the stomach and abdomen the dogs were allowed water on the first day and water and standard dog ration thereafter.

The histamine beeswax suspension that was used was prepared from the following formula: Histaminum dihydrochloride (Imido Hoffmann La Roche) 60 Gm, peanut oil 30.5 cc and white beeswax 3.8 grams. The weighed amount of histamine was ground to a fine powder with a mortar and pestle. Four fifths of the total volume of peanut oil was then gradually mixed in and heated in an oven to 60°C. The beeswax and the remainder of the oil were melted together and added to the histamine mixture. After thorough mixing the suspension was aspirated into heated syringes. Thus the final volume of 36 cc was calculated to contain 100 mg of histamine base per cc of suspension.

The usual dose of histamine beeswax suspension was 30 mg of histamine base per dog given once daily intramuscularly. This is the dosage used by Hay and associates<sup>11</sup>. However, as the incidence of ulcer was rather low in the earlier experiments this dosage was increased two to eight times and given in two daily injections in many instances. Dogs FF through II were deprived of food and water on alternate days to enhance the ulcerogenic effect of histamine.

Three series of studies were performed:

- 1 Control experiments with histamine alone
- 2 Histamine injections begun three weeks after fundic excisional ulcer
- 3 Histamine injections begun three weeks after pyloric excisional ulcer

## RESULTS

*Control Experiments With Histamine Alone*—(See Table I) It may be noted that five out of twelve dogs had pyloric or duodenal ulcers. These ulcers varied in character from multiple acute hemorrhagic ulcers to single chronic, perforating ulcers. Weight loss occurred in all animals in which death was spontaneous. Anorexia and/or vomiting preceded death by one to three days in most cases.

TABLE I INCIDENCE OF ULCERS IN NORMAL DOGS RECEIVING HISTAMINE IN BEESWAX

DOG	SURVIVAL TIME (DAYS)	POST MORTEM FINDINGS		
		FUNDUS	PYLORUS	DUODENUM
JJ	4	Neg	Neg	2 acute ulcers
KH	10	Neg	Neg	2 chronic perf ulcers
LL	8	Neg	Neg	Neg
MM	8	Neg	Neg	Neg
O	7	Neg	Neg	Neg
P	5	Neg	3 acute ulcers	1 acute ulcer
Q	11	Erosions	Neg	Neg
R	7	Neg	2 acute ulcers	Neg
S	12	Neg	Neg	2 acute ulcers
T	5	Neg	Neg	Neg
U	14	Erosive gastritis	Neg	Neg
V	5	Neg	Neg	Neg

Each dog received 30 mg of histamine (base) in beeswax peanut oil once daily. Dog V received 30 mg once daily for thirty days and 30 mg twice daily for twenty three days. Dogs LL, MM and V were sacrificed. The other dogs died.

Summary: Five out of twelve dogs had pyloric and/or duodenal ulcers.



*The Effect of Histamine on 3-Week-Old Fundic Excisional Ulcers*—(See Table II and Fig 1) Four out of five dogs succumbed to ulcers of the duodenum. In each dog the fundic excisional ulcer as well as the gastrostomy were completely healed as evidenced by scar contracture. It is to be considered that histamine-beeswax ulcer seldom, if ever, occurs in the fundus of the stomach.

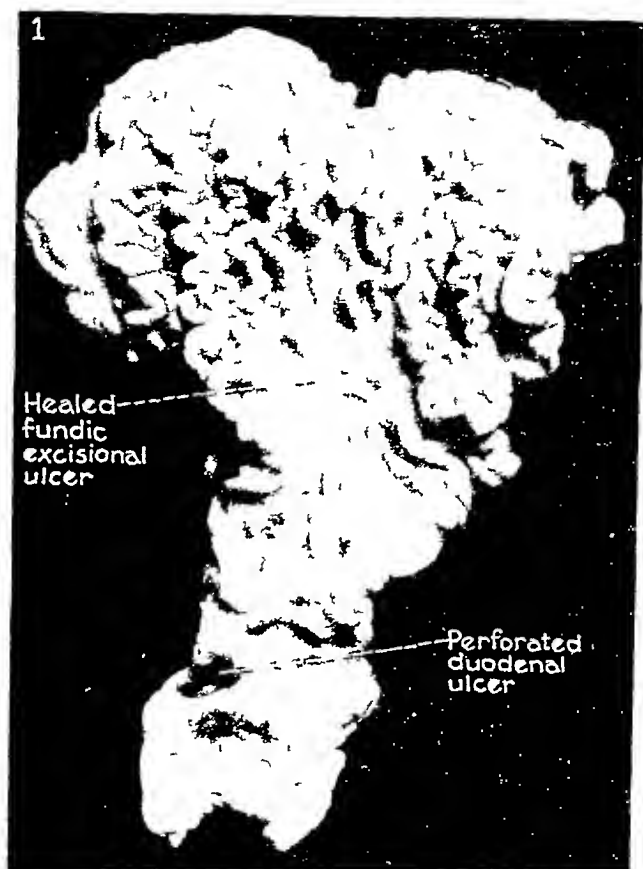


Fig 1—The excisional ulcer of the fundus was made three weeks prior to starting daily injections of histamine in beeswax for three days. The animal died as a result of perforation of the duodenal ulcer.

TABLE II INCIDENCE OF ULCERS IN DOGS IN WHICH DAILY INJECTIONS OF HISTAMINE IN BEESWAX WERE BEGUN THREE WEEKS AFTER EXCISING A PIECE OF FUNDIC MUCOSA

DOG	SURVIVAL TIME (DAYS)	EXCISION ULCER	GASTROS TOMY	FUNDUS	PYLOFUS	DUODENUM
A	4	Healed	Healed	Neg	Neg	5 large nonperf ulcers
B	34	Healed	Healed	Neg	Neg	Severe duodenitis
C	4	Healed	Healed	Neg	Neg	1 large ulcer
D	3	Healed	Healed	Neg	Neg	1 chronic ulcer
E	3	Healed	Healed	Neg	Neg	1 large perf ulcer

Each dog received 30 mg of histamine (base) once daily. Dog B was sacrificed; the remaining dogs died.

Summary: Four out of five dogs had duodenal ulcers. In all cases the excisional ulcer and gastrostomy were completely healed.

*The Effect of Histamine on 3 Week Old Pyloric Excisional Ulcers*—(See Table III and Fig. 2) Seven out of twelve dogs had ulcers of the pylorus and/or duodenum. In every instance the excisional ulcer was completely healed and did not reulcerate as a result of histamine administration. The incomplete healing of two of the gastrostomy wounds was probably due to the use of non absorbable suture material in closing the wound. The fact that ulcerations did occur in areas immediately adjacent to the healed ulcer is strong evidence of the local resistance of the healed ulcer.

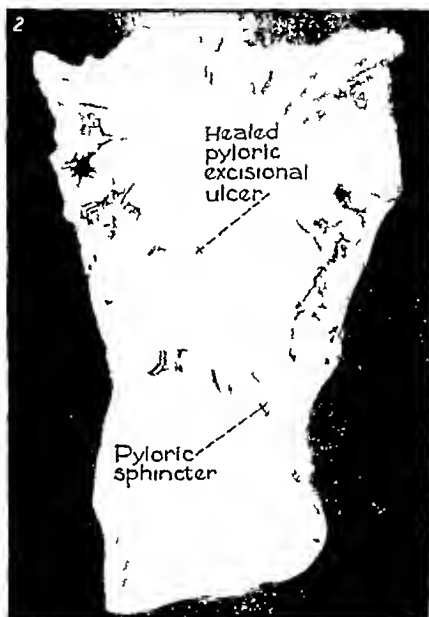


Fig. 2—The excisional ulcer was made three weeks before the daily injections of histamine in beeswax were started. The animal was sacrificed after twelve days of injections because it was moribund. Note that ulcers and erosions occurred immediately adjacent to the healed ulcer but spared the scar itself.

#### DISCUSSION

On mere speculation, a healed ulcer might be expected to be more susceptible than adjacent normal mucosa to injury from histamine and gastric hypersecretion. Certainly present day concepts hold that the 'recurrence' of peptic ulcer in a patient usually represents the breaking down of the old ulcer site rather than the formation of an ulcer at a new site. The evidence of the increased resistance of a recently healed fundic or pyloric excisional ulcer which is provided by the present studies is subject to several interpretations.

TABLE III INCIDENCE OF ULCERS IN DOGS IN WHICH DAILY INJECTIONS OF HISTAMINE IN BEESWAX WERE BEGUN THREE WEEKS AFTER EXCISING A PIECE OF PYLORIC MUCOSA

DOG	SUR VIVAL TIME (DAYS)	EXCISION ULCER	GASTRO- TOMY	FUNDUS	PYLORUS	DUODENUM
F	28	Healed	Healed	Neg	Neg	Neg
G	6	Healed	Healed	Erosions	Erosions	1 acute ulcer
H	28	Healed	Healed	Neg	Neg	Neg
I	28	Healed	Healed	Neg	Neg	Neg
X	55	Healed	Incompletely healed	Neg	3 acute ulcers	Neg
Y	22	Healed	Healed	Neg	1 perf ulcer 1 small ulcer	Neg
Z	28	Healed	Healed	Neg	Neg	Severe duodenitis
EE	1	Healed	Healed	Neg	Neg	Neg
FF	14	Healed	Healed	Erosions	Erosions	Healed perf ulcer
GG	1	Healed	Incompletely healed	Neg	Multiple acute ulcer	Neg
HH	12	Healed	Healed	Neg	Multiple acute ulcer	Neg
II	4	Healed	Healed	Neg	1 large perf ulcer	1 chronic ulcer

Dogs F, H I X Z and HH were sacrificed the remainder died

Summary: Seven out of twelve dogs had ulcers of the pylorus and/or duodenum All excisional ulcers were completely healed

One consideration is that a recently healed ulcer may have cells which are more resistant to damage. This may be due either to an inherent property of the epithelial cells or to the vascularity of the underlying granulation tissue.

The studies of others whose work has already been cited have shown that the fresh unhealed excisional ulcer is more susceptible than the surrounding normal mucosa to histamine induced ulceration. Our studies show that sometime during the healing process this increased susceptibility is reversed, resulting in a greater than normal resistance of the healed ulcer.

It is probable that human peptic ulcers are often not completely healed and so do not reach the stage of increased resistance. If the findings of this study should prove to be applicable to human peptic ulcer disease, they will have important implications for therapy. Thus with vigorous and prolonged ulcer therapy to insure complete healing, the incidence of apparent or real recurrence might be greatly lessened.

#### CONCLUSION

Three-week-old, healed fundic and pyloric excisional ulcers do not break down and reulcerate when histamine-beeswax is given although ulcer formation occurs in the adjacent normal mucosa of the pyloric portion of the stomach and duodenum.

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# CONCENTRATION OF FREE VALINE, TRYPTOPHANE, AND HISTIDINE OF PLASMA OF YOUNG AND OLD INDIVIDUALS, DETERMINED WITH THE MICROBIOLOGIC METHOD

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THE microbiologic method for determining essential amino acids has been used quite frequently for the analyses of protein of various sources<sup>2</sup> with techniques varying as to type of media and bacteria employed.<sup>3</sup> Published data on the free essential amino acids in the blood plasma of normal individuals as determined with the microbiologic technique are still scarce. Hier and Bergem<sup>1</sup> reported the concentrations of ten essential free amino acids in the plasma of thirty-three normal young individuals, nine of them being women. These authors used *Leuconostoc mesenteroides* for the determination of histidine and *Lactobacillus arabinosus* for tryptophane and valine.

The use of more than one type of bacterium requiring also a larger number of media becomes inconvenient whenever the optimal determination of several amino acids is desired. *L. mesenteroides* has been suggested as possibly suitable for the determination of a larger number of amino acids than employed so far, since it was found to require sixteen amino acids for optimal growth.<sup>4</sup> Up to the present, however, we have found no publications in which *L. mesenteroides* was used in the determinations for valine and tryptophane, but it was employed successfully for histidine<sup>1, 4</sup> and others.

We were particularly interested in investigating whether the concentrations of free essential amino acids of the plasma in man are maintained throughout life. Because of the scarcity of data on normal subjects in the literature and as the use of *L. mesenteroides* was extended to the determination of valine and tryptophane our results on old individuals were compared with those on young subjects established in our laboratory with the same techniques.

## METHOD AND MATERIAL

Our subjects included twenty-seven young and twenty-three old individuals. The young subjects were mainly hospital employees who were in good health. The old individuals were inmates of the St. Louis City Infirmary Hospital whose ages ranged from 52 to 88. None of the patients was acutely ill at the time the tests were performed, but all had chronic diseases often found associated with old age. Their clinical diagnoses included generalized arteriosclerosis, arteriosclerotic heart disease, degenerative arthritis, senile psychosis, old cerebral thrombosis, and old bone fractures.

From each of the subjects in the basal state, 50 ml of blood were withdrawn, using heparin as the anticoagulant. The plasma was separated within one hour and tungstic acid filtrates were prepared as described by Hier and Bergem. We used *L. mesenteroides*

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Amino acids courtesy of Merck & Co. Inc. Rahway, N. J.

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for all subjects with the medium D of Dunn and co workers<sup>4</sup> in the determinations for valine, tryptophane and histidine. In addition in fourteen of the young individuals valine and tryptophane was also determined with *I. arabinosus* in the medium described by Henderson and Saelle.<sup>6</sup>

The amino acids and some salts for the two different media were weighed in substance for each determination. Vitamins, purines and the salts A and B made up as described by Dunn and associates<sup>4</sup> were added in solution. The procedure was followed in essentially the same way as described by Dunn and co workers<sup>4</sup> and Stokes and co workers<sup>5</sup> with 1 ml portions of medium for each sample. However for greater accuracy the titrations were carried out potentiometrically. Further after completion of the incubation period, the samples were stored in ice to check further growth during the time required for titrating.

## RESULTS

Table I shows the concentration of valine, tryptophane and histidine expressed in micrograms per milliliter of plasma in young and normal individuals. All the determinations were performed with *I. mesenteroides*.

TABLE I. FREE AMINO ACID CONTENT OF PLASMA IN NORMAL YOUNG INDIVIDUALS  
(REPORTED AS MICROGRAMS PER MILLILITER OF PLASMA)

PATIENT	AGE	VALINE	TRYPTOPHAN	HISTIDINE
Men				
24	37	34.5	10.8	12.0
25	22	30.0	13.8	13.2
26	32	30.0	12.6	15.9
27	28	30.0	13.5	17.7
28	34	29.4	12.3	14.4
29	29	27.0	7.8	
30	25	27.6	11.4	
31	29	31.5	12.9	
32	36	33.0	10.5	
Average		29.3	11.7	14.0
Standard deviation		$\pm 2.2$	$\pm 1.8$	
Women				
33	18	34.8	14.7	14.4
34	38	30.0	15.0	14.1
35	22	30.6	13.8	13.8
36	34	28.2	14.4	13.8
37	32	19.5	11.4	12.3
38	21	21.3	10.5	13.2
39	19	23.7	12.3	14.1
40	28	36.9	11.4	15.0
41	21	30.0	11.7	14.7
42	19	27.9	8.7	
43	20	22.5	9.3	
44	21	27.0	9.0	
45	21	33.7	13.2	
46	20	26.4	12.3	
47	21	24.9	12.3	
48	21	28.8	12.9	
49	21	22.8	6.9	
50	21	31.2	12.6	
Average		27.8	11.8	13.9
Standard deviation		$\pm 4.6$	$\pm 2.2$	
Total				
Average		29.6	11.8	14.2
Standard deviation		$\pm 4.0$	$\pm 2.1$	$\pm 1.3$

TABLE II COMPARISON OF THE CONCENTRATION IN THE PLASMA OF FREE VALINE AND TRYPTOPHANE DETERMINED WITH *L* MESENTEROIDES AND *L* ARABINOSIS IN FOURTEEN YOUNG INDIVIDUALS (REPORTED IN MICROGRAMS PER MILLILITER OF PLASMA)

PATIENT	SEX	AGE	VALINE		TRYPTOPHANE	
			<i>L</i> MESENTEROIDES	<i>L</i> ARABINOSIS	<i>L</i> MESENTEROIDES	<i>L</i> ARABINOSIS
29	M	25	27.0	29.4	7.8	8.1
30	M	25	27.6	27.0	11.4	12.0
31	M	25	31.5	34.2	12.9	13.5
42	F	19	27.9	30.0	8.7	9.3
32	M	36	33.0	33.9	10.5	9.6
38	F	21	21.3	21.9	10.5	10.8
43	F	20	22.5	24.6	9.3	11.4
44	F	21	27.0	26.4	9.0	9.0
45	F	21	33.7	36.1	13.2	13.8
46	F	20	26.4	27.6	12.3	11.4
47	F	21	24.9	26.4	12.3	11.7
48	F	21	28.8	31.8	12.9	12.9
49	F	21	22.8	24.3	6.9	6.9
50	F	21	31.2	31.5	12.6	13.2
Mean			27.5	28.9	10.7	11.0
Standard deviation			±3.7	±4.0	±2.0	±2.0

Parallel studies with *L mesenteroides* and *L arabinosis* for valine and tryptophane showed good agreement, as may be noted in Table II. Small differences found in some of the determinations were within the error limit. The differences of the means were not significant, although the determinations with *L arabinosis* yielded slightly higher values for valine. We found, however, better agreement on various levels of the slope in the determinations that were carried out with *L mesenteroides* than with those with *L arabinosis*.

The concentrations of free valine, tryptophane, and histidine in old individuals determined with *L mesenteroides* are recorded in Table III.

TABLE III FREE AMINO ACID CONTENT IN ELDERLY PEOPLE

PATIENT	SEX	AGE	VALINE ( $\mu\text{G}/\text{ML}$ )	TRYPTOPHANE ( $\mu\text{G}/\text{ML}$ )	HISTIDINE ( $\mu\text{G}/\text{ML}$ )
1	F	68	32	13.2	18.3
2	F	82	22.8	10.8	15.0
3	F	52	19.6	12.3	16.5
4	M	74	25.5	9.9	13.2
5	M	73	23.7	12.6	18.3
6	M	71	24.9	9.0	11.1
7	M	74	24.6	12.6	16.2
8	M	68	25.2	12.0	13.8
9	M	68	24.9	10.2	15.3
10	M	81	21.3	8.1	13.8
11	M	88	21.3	9.0	13.2
12	F	83	24.0	10.5	12.9
13	M	77	27.9	8.1	13.8
14	M	81	23.4	9.6	14.1
15	M	72	22.5	9.9	14.1
16	M	71	25.2	7.8	11.1
17	M	66	28.5	10.2	11.7
18	M	60	18.9	8.4	12.9
19	M	66	19.2	5.7	8.7
20	M	67	25.2	9.9	15.6
21	M	90	27.3	9.9	14.1
22	M	75	24.6	10.5	14.1
23	F	69	22.8	13.2	14.4
Average			24.6	10.2	14.0
Standard deviation			±3.1	±1.7	±2.2

## DISCUSSION

The results obtained in young people with *L. mesenteroides* for valine and tryptophane compare well with those obtained in the literature that were carried out with *L. arabinosis*. They were somewhat higher in the men for valine (see Table IV), the mean being  $29.6 \pm 4$   $\gamma$  per milliliter while Hies and Bergem reported  $28.3 \pm 3.4$   $\gamma$  per milliliter. The concentration of tryptophane averaged for the young group  $11.8 \pm 2.1$   $\gamma$  per milliliter, compared with the literature value of  $10.8 \pm 2.1$   $\gamma$  per milliliter. Histidine determined with the same bacterium gave a mean identical with that obtained by Hies and Bergem of  $14.2 \pm 2.2$   $\gamma$  per milliliter.

TABLE IV. COMPARISON OF RESULTS OF AMINO ACIDS OF OUR LABORATORY WITH THOSE OF HIES AND BERGEM (REPORTED AS MICROGRAMS PER MILLILITER OF PLASMA)

AMINO ACID	OUR LABORATORY			HIES AND BERGEM		
	NUMBER OF PATIENTS	AVERAGE	STANDARD DEVIATION	NUMBER OF PATIENTS	AVERAGE	STANDARD DEVIATION
Valine						
Men	9	30.3	$\pm 2.2$	24	28.0	$\pm 3.7$
Women	18	27.8	$\pm 4.6$	9	26.7	$\pm 3.6$
Total	27	29.6	$\pm 4.0$	33	28.3	$\pm 3.4$
Tryptophane						
Men	9	11.7	$\pm 1.8$	24	11.1	$\pm 2.3$
Women	18	11.8	$\pm 2.2$	9	9.8	$\pm 1.1$
Total	27	11.8	$\pm 2.1$	33	10.8	$\pm 2.1$
Histidine						
Men	5	14.6		24	14.2	$\pm 2.4$
Women	9	13.9		9	14.0	$\pm .8$
Total	14	14.2	$\pm 1.3$	33	14.2	$\pm 1.8$

A comparison of the concentrations of young and old individuals (Table V) disclosed no difference for histidine  $14.2$   $\gamma$  per milliliter compared with  $14.0 \pm 2.2$   $\gamma$  per milliliter, a not significant difference for tryptophane,  $11.8$   $\gamma$  per milliliter compared with  $10.2$   $\gamma$  per milliliter, but a definitely lower value

TABLE V. COMPARISON OF AMINO ACID CONTENT OF PLASMA IN YOUNG AND OLD PEOPLE (REPORTED IN MICROGRAMS PER MILLILITER OF PLASMA)

	NUMBER OF PATIENTS	VALINE	NUMBER OF PATIENTS	TRYPTOPHANE	NUMBER OF PATIENTS	HISTIDINE
Old people	23	24.6 $\pm 3.1$	23	10.2 $\pm 2.1$	23	14.0 $\pm 2.2$
Young people	27	29.6 $\pm 4.0$	27	11.8 $\pm 2.1$	14	14.1 $\pm 1.4$
Difference		5.0		1.6		1
Standard error		$\pm 1.0$		$\pm .6$		

for valine. The mean for valine in the young normal subjects was  $29.6 \pm 4$   $\gamma$  per milliliter, while it comprised in the old individuals only  $24.6 \pm 3.1$   $\gamma$  per milliliter, the difference being  $5$   $\gamma$  per milliliter of plasma. The standard error was calculated<sup>9</sup> and found to be  $1.0$   $\gamma$  per milliliter.

## SUMMARY

1. The results obtained with *L. mesenteroides* in determining tryptophane and valine are comparable with those determined with *L. arabinosis*.



TABLE II AGENIZED PROTEIN SUPPLEMENTS TO ADULTS' NORMAL DIET OF 2,500 3,000 CALORIES PER DAY

GLUTEN CEREAL		CHOCOLATE MILK	
100 Gm	cooked cereal	1 Glass	milk
15 Gm	gluten (225 Gm Agene per ewt)	1 Tablespoon	chocolate syrup
25 Gm	hot water	25 Gm	casein (20 Gm Agene/ewt)
GLUTEN MUFFINS (Makes 12 muffins)			
	1 Yeast cake		
	1 Cup milk		
	1 Teaspoon salt		
	2 Teaspoons butter		
	1 Cup bread flour (20 Gm Agene/ewt)		
	190 Gm gluten (225 Gm Agene/ewt)		
	1 Slightly beaten egg white		
1 Helping cereal	15 Gm gluten	12 5 Gm	protein
1 Muffin	15 Gm gluten	12 5 Gm	protein
	15 Gm flour	1 9 Gm	protein
1 Glass choc milk	25 Gm casein	23 7 Gm	protein
Each adult patient regularly consumed			
		1 Helping cereal	
		1 Muffin	
		3 Glasses chocolate milk	
each day as a supplement to normal diet (98 Gm Agenized protein)			

within normal limits. These subjects had noted no untoward symptoms while on the diet. One subject gained almost thirty pounds and the other ten pounds in weight during the experimental period.

#### DISCUSSION OF RESULTS

Previous experiments have shown that the greater the amount of Agene treated material in the ration or the more heavily it is treated the more rapid is the onset of the gross and electroencephalographic abnormalities in dogs, Newell and co-workers<sup>4</sup>. One would expect the same to be true in man if he were susceptible to a possible toxic factor in the Agenized material.

Flour bought on the market is usually treated with 1.25 to 2.5 Gm of Agene per hundredweight. Not only were the experimental diet supplementations more heavily treated with Agene, but also as much as 100 Gm of highly Agenized protein (225 Gm Agene per hundredweight) were consumed in addition to the normal diet which contained wheat products treated with commercial levels of Agene. We suggest from these facts that, by analogy with the rations used to produce canine epilepsy, the diets used in these human observations should be an adequate test of the susceptibility of man to a similar toxic factor in Agenized material.

None of the nineteen patients (Table III) who consumed this highly Agenized diet showed any clinical or electroencephalographic evidence of the development of epilepsy. Nor did the five epileptic patients show any demonstrable increase in the number of epileptic seizures or any significant increase in the abnormality present in their electroencephalograms.

## DISCUSSION

The results obtained in young people with *L. mesenteroides* for valine and tryptophane compare well with those obtained in the literature that were carried out with *L. arabinosus*. They were somewhat higher in the men for valine (see Table IV), the mean being  $29.6 \pm 4$   $\gamma$  per milliliter while Hier and Bergheim reported  $28.3 \pm 3.4$   $\gamma$  per milliliter. The concentration of tryptophane averaged for the young group  $11.8 \pm 2.1$   $\gamma$  per milliliter, compared with the literatures' value of  $10.8 \pm 2.1$   $\gamma$  per milliliter. Histidine determined with the same bacterium gave a mean identical with that obtained by Hier and Bergheim of  $14.2 \pm 2.2$   $\gamma$  per milliliter.

TABLE IV. COMPARISON OF RESULTS OF AMINO ACIDS OF OUR LABORATORY WITH THOSE OF HIER AND BERGHEIM (REPORTED AS MICROGRAMS PER MILLILITER OF PLASMA)

AMINO ACID	OUR LABORATORY			HIER AND BERGHEIM		
	NUMBER OF PATIENTS	AVGAGE	STANDARD DEVIATION	NUMBER OF PATIENTS	AVGAGE	STANDARD DEVIATION
Valine						
Men	9	30.7	$\pm 2.2$	24	28.0	$\pm 3.7$
Women	19	27.8	$\pm 4.6$	9	26.7	$\pm 3.6$
Total	27	29.6	$\pm 4.0$	33	28.3	$\pm 3.4$
Tryptophane						
Men	9	11.7	$\pm 1.8$	24	11.1	$\pm 2.3$
Women	18	11.8	$\pm 2.2$	9	9.8	$\pm 1.1$
Total	27	11.8	$\pm 2.1$	33	10.8	$\pm 2.1$
Histidine						
Men	5	14.6		24	14.2	$\pm 2.4$
Women	9	13.9		9	14.0	$\pm .8$
Total	14	14.2	$\pm 1.3$	33	14.2	$\pm 1.8$

A comparison of the concentrations of young and old individuals (Table V) disclosed no difference for histidine  $14.2$   $\gamma$  per milliliter compared with  $14.0 \pm 2.2$   $\gamma$  per milliliter. A not significant difference for tryptophane,  $11.8$   $\gamma$  per milliliter compared with  $10.2$   $\gamma$  per milliliter but a definitely lower value

TABLE V. COMPARISON OF AMINO ACID CONTENT OF PLASMA IN YOUNG AND OLD PEOPLE (REPORTED IN MICROGRAMS PER MILLILITER OF PLASMA)

	NUMBER OF PATIENTS	VALINE	NUMBER OF PATIENTS	TRYPTOPHANE	NUMBER OF PATIENTS	HISTIDINE
Old people	23	24.6 $\pm 3.1$	23	10.2 $\pm 2.1$	23	14.0 $\pm 2.2$
Young people	27	29.6 $\pm 4.0$	27	11.8 $\pm 2.1$	14	14.1 $\pm 1.4$
Difference		5.0		1.6		1
Standard error		$\pm 1.0$		$\pm .6$		

for valine. The mean for valine in the young normal subjects was  $29.6 \pm 4$   $\gamma$  per milliliter, while it comprised in the old individuals only  $24.6 \pm 3.1$   $\gamma$  per milliliter, the difference being  $5$   $\gamma$  per milliliter of plasma. The standard error was calculated<sup>9</sup> and found to be  $1.0$   $\gamma$  per milliliter.

## SUMMARY

1 The results obtained with *L. mesenteroides* in determining tryptophane and valine are comparable with those determined with *L. arabinosus*.

2 Determinations of free valine and tryptophane of the plasma in twenty-seven normal young individuals yielded means of  $29.6 \pm 4.0$   $\gamma$  per milliliter and  $11.8 \pm 2.1$   $\gamma$  per milliliter respectively. This compares well with findings reported previously by other authors.

3 The concentrations of free tryptophane and histidine in the blood plasma of elderly individuals were found not to differ significantly from those of young normal subjects while the concentration of free valine is lower in old individuals, at least in those who are afflicted with degenerative diseases.

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# STUDIES ON HUMAN SUBJECTS RECEIVING HIGHLY AGENIZED FOOD MATERIALS

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FOR over a quarter of a century nitrogen trichloride (commonly known as Agene) has been used by the milling industry as a maturing agent for white flour. The apparent superiority of Agene over other agents and the ease with which this gaseous component can be handled have made it the most common maturing agent for white flour.

In December of 1946 Mellanby<sup>1</sup> first reported that running fits could be precipitated in dogs when they were fed diets containing flour treated with nitrogen trichloride. As far back as 1937, Melnick and Cowgill<sup>2</sup> reported that the alcohol soluble moiety of wheat protein, gliadin caused convulsions when fed to dogs. Wagner and Elvehjem<sup>3</sup> in 1944 reported that wheat gluten, the water insoluble portion of wheat protein produced running fits when added to an otherwise complete ration. As little as 10 per cent wheat gluten in a purified ration induced these attacks in twenty five to thirty days while increased amounts of the protein brought on seizures in proportionally shorter periods. Further investigation by Newell and co-workers<sup>4</sup> after Mellanby's report showed that Agene but none of the other commercial maturing and/or bleaching agents would react with flour or gluten to produce the toxic factor which caused fits. In order to induce fits readily in dogs flour was included at 84 per cent of the ration.

The American diet rarely contains more than 30 per cent of flour. Five dogs were placed on rations which contained 30 per cent of flour treated with commercial levels of Agene. These animals were fed the rations for periods of from ten to twelve months and all remained healthy with no indication of running fits. Electroencephalograms were taken on these animals after they had been on experiment for seven to nine months and all showed normal tracings. Thus it was apparent that in order to produce any effect in dogs it was necessary to feed the commercially treated flour at excessive levels or the flour had to be treated with large quantities of Agene.

The susceptibility of other species of animals has been tested, and while none were quite as sensitive as the dog, the convulsive state was readily induced by feeding highly Agenized flour rations to rabbits, cats, mink, and

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ferrets No effect was observed when Agerized rations were fed to rats, chicks, or guinea pigs Monkeys fed highly Agerized flour rations showed abnormal electroencephalograms, but no gross changes Silver and associates<sup>6</sup> also reported abnormal electroencephalograms and stated that monkeys fed highly Agerized rations developed asynergy, weakness of the extremities, and sluggishness in response to irritating stimuli in comparison with normal animals Radomski and co-workers,<sup>7</sup> however, observed no adverse conditions when monkeys were fed highly Agerized rations for as long as fifty-four days

It was reported in 1946<sup>8</sup> that electroencephalograms taken on dogs fed Agerized wheat gluten showed brain wave patterns similar to those seen in cases of idiopathic epilepsy in man Since the convulsions and electroencephalographic patterns of these dogs showed a close analogy to human epilepsy the question arose whether Agerized wheat products play any part in the incidence of human epilepsy The publication of Mellanby's article at this time added emphasis to the problem Articles have appeared in the lay press implying that Agerized flour is an important cause of human epilepsy even though no direct evidence has been published to support such statements

In an effort to answer this question, children and adults have been fed diets which contain normal and excessive amounts of Agerized food products

#### METHODS AND RESULTS

The children, 5 to 16 years of age, were hospital patients, the adults were students at the University Some subjects had no previous history of epilepsy, while five patients had had seizures from time to time After a physical examination by a member of the medical staff each patient was placed on a normal diet for a week or more, during which time several control electroencephalograms (EEG) were taken After the characteristic electroencephalogram of each patient had been determined, supplements of white flour, whole wheat flour, wheat gluten and casein treated with Agene (3 to 225 grams per hundredweight) were incorporated into each meal fed the patients The foods treated with high levels of Agene were included in such items as cereal, chocolate milk, muffins, bread, gravy, and meat patties Although the patients knew an experimental diet was under test, they did not know what was being used or what effect the diet might have upon them Electroencephalograms were taken every three to four days while the Agerized diet was fed Some of the subjects were continued on the diet for periods as long as 210 days The children were offered 100 to 150 Gm of Agerized food products per day, but the consumption was not constant due to the unpalatability of the food supplements On the whole, the children consumed 65 to 75 per cent of the Agerized foods offered The adults were fed Agerized foods on the basis of their protein content and, in general, attempts were made to include sufficient quantities of the treated materials to supply 100 Gm per day Since palatability had been increased as a result of previous tests in feeding the chil-

dren and because adults were easier to work with, all the Agenized foods offered were consumed by these subjects except two patients (that is C M and M B) who developed nausea.

In Table I are summarized the Agenized food supplements offered to the children. Consumption in terms of Agenized protein ranged from 40 to 114 Gm per day depending upon the acceptance of the foodstuffs by the patients. The flour was treated with 3 Gm of Agene per hundred pounds which is two times the amount of Agene used to treat flour commercially. The gluten was made from flour treated with 4 Gm of Agene per hundred pounds (which is equivalent to 40 Gm of Agene per hundred pounds of gluten). Thus the children received daily twenty three to thirty times as much active material as might be present in a normal diet.

TABLE I AGENIZED FOOD SUPPLEMENTS TO CHILDREN'S NORMAL DIET OF 1500-1800 CALORIES PER DAY

GLUTEN CEREAL		CHICKEN MEAT Patties	
100 Gm cooked cereal		75 Gm raw ground meat	
25 Gm gluten (40 Gm Agene per cwt)		25 Gm gluten (40 Gm Agene per cwt)	
25 Gm hot water		Milk to moisten	
GLUTEN MUFFINS (Makes 12 muffins)			
	1 Yeast cake		
	1 Cup milk		
	1 Teaspoon salt		
	2 Teaspoon butter		
	1 Cup bread flour (3 Gm Agene/cwt)		
	180 Gm gluten (40 Gm Agene/cwt)		
	1 Slightly beaten egg white		
1 Helping cereal	25 Gm Gluten	21 Gm protein	
1 Meat patty	25 Gm gluten	21 Gm protein	
1 Muffin	15 Gm gluten	12.5 Gm protein	
	15 Gm flour	19 Gm protein	
Each child fed	1 Helping cereal		
	1 Meat patty		
	3.5 Muffins		
each day as a supplement to normal diet (80-114 Gm Agenized protein)			

Table II presents a summary of the Agenized protein supplements consumed by the adults while on the experimental diet. All subjects ate about 100 Gm of highly Agenized protein (that is treated with 20 to 225 Gm Agene per hundredweight) per day. The Agene treatment of the material fed to human subjects of the present experiment was twenty two times more than that of the normal diet. A total of nineteen patients have been tested thus far and the results are presented in Table III.

In addition two of the adults (R G H and A D S) were subjected to more intensive examinations after they had been on the Agene diet for a period of seven months. The gastrointestinal x-ray study was completely negative. Erythrocytes, leucocytes, and differential blood counts as well as urinalysis, blood sugar, nonprotein nitrogen, and serum protein values were

TABLE II AGENIZED PROTEIN SUPPLEMENTS TO ADULTS' NORMAL DIET OF 2,500 3,000 CALORIES PER DAY

GLUTEN CEREAL		CHOCOLATE MILK	
100 Gm	cooked cereal	1	Glass milk
15 Gm	gluten (225 Gm Agene per cwt)	1	Tablespoon chocolate syrup
25 Gm	hot water	25 Gm	casein (20 Gm Agene/cwt)
GLUTEN MUFFINS (Makes 12 muffins)			
	1 Yeast cake		
	1 Cup milk		
	1 Teaspoon salt		
	2 Teaspoons butter		
	1 Cup bread flour (20 Gm Agene/cwt)		
180 Gm	gluten (225 Gm Agene/cwt)		
	1 Slightly beaten egg white		
1 Helping cereal	15 Gm gluten	12.5 Gm	protein
1 Muffin	15 Gm gluten	12.5 Gm	protein
	15 Gm flour	1.9 Gm	protein
1 Glass choc milk	25 Gm casein	23.7 Gm	protein
Each adult patient regularly consumed			
		1	Helping cereal
		1	Muffin
		3	Glasses chocolate milk
each day as a supplement to normal diet (98 Gm Agenized protein)			

within normal limits. These subjects had noted no untoward symptoms while on the diet. One subject gained almost thirty pounds and the other ten pounds in weight during the experimental period.

#### DISCUSSION OF RESULTS

Previous experiments have shown that the greater the amount of Agene treated material in the ration or the more heavily it is treated the more rapid is the onset of the gross and electroencephalographic abnormalities in dogs, Newell and co-workers<sup>4</sup>. One would expect the same to be true in man if he were susceptible to a possible toxic factor in the Agenized material.

Flour bought on the market is usually treated with 1.25 to 2.5 Gm of Agene per hundredweight. Not only were the experimental diet supplementations more heavily treated with Agene, but also as much as 100 Gm of highly Agenized protein (225 Gm Agene per hundredweight) were consumed in addition to the normal diet which contained wheat products treated with commercial levels of Agene. We suggest from these facts that, by analogy with the rations used to produce canine epilepsy, the diets used in these human observations should be an adequate test of the susceptibility of man to a similar toxic factor in Agenized material.

None of the nineteen patients (Table III) who consumed this highly Agenized diet showed any clinical or electroencephalographic evidence of the development of epilepsy. Nor did the five epileptic patients show any demonstrable increase in the number of epileptic seizures or any significant increase in the abnormality present in their electroencephalograms.

TABLE III CLINICAL HISTORIES AND RESULTS OF FEEDING HIGHLY AMINIZED DIETS TO HUMAN SUBJECTS

NAME	SEX	AGE (yr)	DIAGNOSIS	COGITROL FEEDING	TIME ON DIET (DAYS)	RESULT
L. A. L.	M	19	Normal	6/sec and 8 12/sec waves	60	No change
M. R. A.	F	22	Grand mal, petit mal epilepsy	Variable normal, 1 5 6/sec waves	59	No convulsions, "Much better", gained wt, EEG variable, between normal, F <sub>1</sub> spike and wave
J. M. B.	M	18	Allergic dermatitis	Normal 8 12/sec waves	12	No change
M. B.	F	24	Idiopathic epilepsy dermatitis	Normal 8 12/sec waves	12	Nausea, no change
M. E. B.	M	19	Hypertrophied tonsils	Normal 8 12/sec waves	64	No change
J. V. F.	M	9	Fracture of right hip	Normal for age	21	No change
D. G.	M	7	Pertiles' disease	Normal for age	28	No change
W. A. G.	M	19	Myopia, acne, grand mal epilepsy	Normal 8 12/sec waves	59	No change
E. H.	F	8	Bronchiectasis mental deficiency	Normal for age	21	No change
R. G. H.	M	17	Myopia	Normal 8 12/sec waves	210	No change
T. J. L.	M	21	Impetigo	Normal 8 12/sec waves	59	No change
V. M. M.	F	10	Congenital dislocation of hip, acute cystitis	Normal for age	14	No change
E. R. R.	M	4	Epilepsy due to focal cerebral cicatrix	Normal for age	12	No convulsions
W. F. R.	M	11	Epilepsy, grand mal, porencephaly, mental retardation	1 5 3/sec waves and spikes	7	No change
G. E. S.	F	20	No disease	Normal 8 12/sec waves	15	No change
M. F. T.	M	23	No disease	Variable 8 12 and 3 5 8/sec waves	7 14	EEG varied independently of diet
S. G. T.	F	10	Mental deficiency	Variable 3 6/sec waves	19	EEG still variable no marked change
A. E. S.	M	20	No disease	Normal, 8 12/sec waves	210	No change in EEG or clinical condition except gain in weight
C. M. M.	M	22	No disease	Normal, 8 12/sec waves	25	No change, lost weight



Susceptibility to the development of epilepsy from the possible toxic effect of the Agenized material would be expected to vary. One patient (M F T) (Table III) who was clinically normal, having no history of epileptiform seizures, had periodic bursts of slow waves suggesting that he had the genetic or constitutional background for the development of seizures. Since he seemed to be an ideal subject for testing the effect of the diet, he was kept under observation for nine months and was placed on the Agene diet for two separate periods. There was no consistent relationship between his abnormal electroencephalogram and the time on the diet. At no time did he develop any suggestion of epileptic seizures though from the appearance of his electroencephalogram one would have expected him to be especially susceptible. The lack of effect of the Agene diet on the normal individuals, those with unrelated illness, and those with epilepsy in the present experiments does not eliminate the possibility that there may be some patients with an idiosyncrasy or an allergy to the Agenized products. Perhaps patients with a known allergy for wheat products should be investigated further in this manner as should those with subclinical epilepsy.

The question arises whether changes would be observed if the Agene diet were continued over a longer period of time. By comparison with dogs in which the characteristic electroencephalographic abnormality and convulsions appear within three or four days it is evident from Table III that the duration of the trial has been adequate. It has been intimated that a lifetime consumption of bread made from flour treated with Agene may cause disturbance of the nervous system and might induce such things as duodenal ulcers (Silver and co-workers<sup>6</sup>). It might be difficult to disprove such a theory as far as the other diseases mentioned by these authors are concerned, but it is well known that epilepsy has been common since the dawn of history (Temkin<sup>9</sup>). There is no evidence of an increase in the incidence of epilepsy in the past quarter of a century during which the use of Agenized flour has become widespread. As noted previously, in our patients (A E S and R G H) there were no clinical symptoms of gastric or duodenal ulcers, and the gastrointestinal x-ray examinations were negative after the patients had been on the diet for seven months.

The negative results of this study in human beings confirms the species specificity of the toxic effects of Agenized protein indicated by Newell and associates,<sup>4</sup> that is, positive results were obtained in dogs, cats, and rabbits, and no effect was observed in rats, chicks, or guinea pigs. The effect on monkeys was rather doubtful. This variation in the response of the different species suggests a difference in the metabolism of the material in these different animals. Possibly there is preferential absorption or a detoxification mechanism present in man and the other nonsusceptible animals which is not present in the dog. Analogous metabolic differences are well known.

#### SUMMARY

Nineteen patients, including five with epilepsy, have been fed a diet containing twenty-two to thirty times the amount of Agenized material contained

in a normal diet. Physiological, neurological and electroencephalographic examinations have failed to disclose any abnormal changes as the result of such a diet and none of the patients has developed epileptic seizures as a result of this diet. The results are discussed in relation to previous studies which have shown that Acnized protein produces an epileptic condition (running fits) in dogs and other animals. It is suggested that this species difference in response to Acnized material represents a species difference in absorption or detoxifying mechanisms.

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## A COMPARISON OF THE BROMSULFALEIN AND ROSE BENGAL TESTS

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DYE excretion tests have been used for over twenty-five years as an index of liver function. Today the bromsulfalein test as introduced by Rosenthal and White<sup>1</sup> and modified by Mateer and co-workers<sup>2</sup> and the rose bengal test as done by Althausen, Biskind, and Keri<sup>3</sup> are the most widely used to measure hepatic reticulo-endothelial activity. The bromsulfalein\* test has been subjected to numerous modifications, but it is generally accepted that the administration of 5 mg per kilogram intravenously as first suggested by Macdonald<sup>4</sup> has increased the sensitivity of the test. Conservative normal values for forty-five and sixty minute specimens are 5 and 2 per cent respectively and are in accord with the work of Mateer, who designates 4 per cent as the upper normal for the retention at forty-five minutes. We have accepted a slightly higher value for our forty-five minute specimen so as to eliminate all possibility of false positive results. We have found that when a curve is prepared on semilogarithmic paper from accurately timed specimens taken from normal patients, the resulting graph closely approximates a straight line until very low values are reached (below 1 per cent). Several such curves are shown in Fig 1. It can be noted that the occasional line which reaches 1 per cent at the end of an hour will pass close to 4 per cent in forty-five minutes. For this reason, a slightly higher normal value is used for the forty-five minute sample and a confirmatory sample is drawn at sixty minutes.

The rose bengal test is performed by the intravenous administration of 10 cc of a 1 per cent solution of diiodotetrachlorofluorescein. A two minute sample is withdrawn and represents the 100 per cent concentration. An eight and sixteen minute sample are taken and the amount of the dye retained is determined by the hand spectrophotograph. Althausen and co-workers give 55 and 35 per cent respectively as the upper limits of normal for the two samples.

The rose bengal test was developed in an era when the 2 mg test of Rosenthal and White was in vogue and was considered to be of comparable sensitivity and perhaps of greater specificity. The series here presented was done in an effort to determine whether the rose bengal test is as sensitive as the 5 mg test of Mateer and associates.

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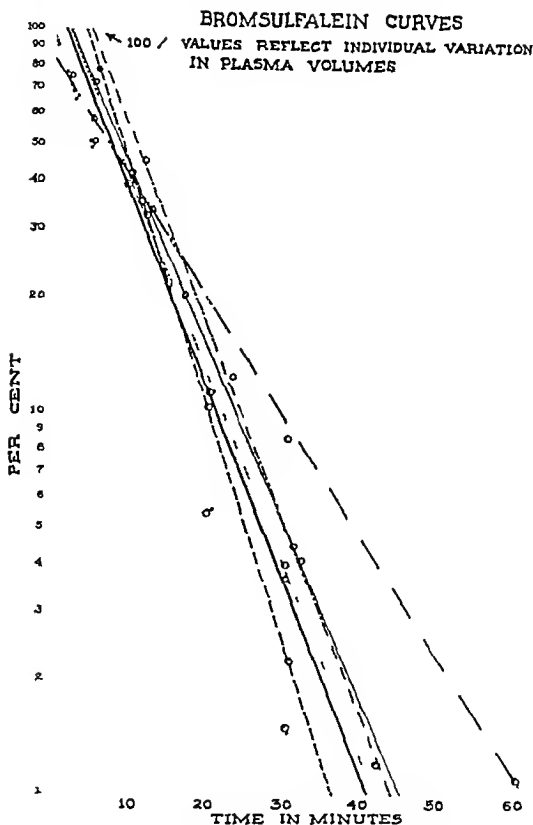


Fig 1 --Bromsulfalein disappearance in normal subjects

#### METHODS

The bromsulfalein concentrations were determined by the use of the Evelyn photo electric colorimeter after the method described by Bradley<sup>5</sup>. The rose bengal determinations were done after the method of Althausen and associates<sup>3</sup> using the hand spectrograph. The patients used in this series were all suspected of having liver dysfunction and many had clinical evidence of hepatic impairment.

#### RESULTS

The results of this study are found in tabular form in Table I.

TABLE I

NO	DIAGNOSIS	BROMSULFALIN		ROSE BENGAL		INTERVAL
		45 MIN	60 MIN	8 MIN	16 MIN	
1	Portal cirrhosis	14	11	78	50	5 wk
2	Portal cirrhosis	7	5	49	20	2 wk
3*	Portal cirrhosis	11	6	41	19	6 wk
4*	Portal cirrhosis	17	15	41	20	2 days
5*	Probable fatty liver	9	5	50	20	1 wk
6*	Probable fatty liver	8	6	54	28	5 wk
7	Post hepatitis	5	2	42	22	2½ wk
8	Portal cirrhosis	40	31	60	36	1 wk
9	Syphilis, hepatomegaly	12	8	60	38	3 days
10*	Portal cirrhosis	10	6	46	23	4 wk
11*	Portal cirrhosis	42	35	54	35	10 days
12	Portal cirrhosis	26	21	77	51	2 wk
13	Probable hemochromatosis	29	26	74	64	4 days
14*	Post hepatitis	7	4	40	22	2½ wk
15	Hemochromatosis	3	1	53	24	2 wk
16*	Toxic cirrhosis	21	5	37	21	1 wk
17	Portal cirrhosis	35	25	69	56	4 wk
18	Hepatoma, autopsy	22	20	57	36	2 days
19*	Probable portal cirrhosis	32	24	45	23	2 days
20	Diabetes	5	2	42	21	4 days
21	Leucemia	5	2	49	29	5 days
22	Hepatomegaly	1	1	52	38	2½ wk
23*	Probable portal cirrhosis	9	5	54	28	6 wk
24	Hepatomegaly	1	1	43	20	7 wk

\*Denotes patients with positive BSP and negative rose bengal tests

### DISCUSSION

Of the twenty-four patients suspected of having liver dysfunction, ten (41.7 per cent) had positive BSP tests and negative rose bengal determinations. One patient (No 22) had a positive value for the sixteen minute sample of rose bengal—the eight minute sample and the BSP test were normal. There were several patients e.g. Nos 18 and 8, who showed a strongly positive BSP and borderline positive rose bengal values.

Technically the BSP is the simpler of the two tests. The readings as obtained on the Evelyn photoelectric colorimeter have little element of individual error and are not significantly influenced by hemolysis. Bromsulfalein has several disadvantages. It is expensive. Moreover, unlike rose bengal, it is not eliminated entirely by the liver and spillage occurs via the kidney when high blood levels are encountered, thereby tending to falsify the test in favor of better than actual function in cases with severe pathologic retention. When BSP is extravasated, it can cause a severe chemical cellulitis with the appearance of brown swelling and violaceous coloration.

Rose bengal is relatively cheap and has the advantage of specificity, but also has some disadvantages. The patients must be warned to stay out of direct sunlight after the test because photosensitivity is apt to occur. In addition cells hemolyze *in vitro* in the presence of rose bengal when they are exposed to direct sunlight. The appearance of red stools after the test although frightening to the patient cannot be deemed a disadvantage. The visual spectrophotographic matching of the absorption bands in the determination

of rose bengal is sometimes difficult particularly when bands of hemoglobin are present in sufficient strength to interfere. The last most important difficulty might perhaps be eliminated by analysis with a spectrophotometer.

This series can be criticized because of the fact that liver function might have changed during the period which elapsed during the performance of the tests. However, a large discrepancy favoring the BSP test existed when the performance of the two tests was only a few days apart. Further this tendency was not influenced by doing one test or the other first, the series being about equally divided in this respect. The lack of correlation between the two tests and the consistency with which the BSP was positive in the presence of a negative rose bengal is a strong indication that the BSP is a more sensitive procedure. However the sensitivity of a test does not necessarily reflect its utility and further work may clarify the relative value of these procedures.

#### SUMMARY

A series of twenty four patients with suspected liver disease is presented in which the bromsulphalein and rose bengal tests are compared. Forty one and seven tenths per cent of the patients with normal rose bengal tests had positive BSP determinations. The 5 mg BSP test appears to be more sensitive than the rose bengal test as determined by the visual spectriograph.

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## OBSERVATIONS ON THE HISTAMINE CONTENT OF THE CEREBROSPINAL FLUID IN MAN

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IT IS generally agreed by most workers that 80 to 90 per cent of the blood histamine content is held within the white cell elements,<sup>1,2</sup> but there is considerable controversy as to which of the white cells carries this bound or intracellular histamine. Some believe that the polymorphonuclear eosinophile is responsible,<sup>3</sup> whereas others consider that the platelets are mainly involved.<sup>4</sup>

Since cerebrospinal fluid may contain polymorphonuclear or lymphocytic cells as a result of meningeal irritation, it was thought that a study of the histamine content of such cerebrospinal fluid, as well as that of normal samples, might provide some evidence as to which type of cell is responsible for carrying histamine.

### METHODS

Cerebrospinal fluid collected by lumbar puncture in the case of normal subjects, or directly from the ventricle as well as by tap in the neurosurgical patients, was extracted for histamine by the method described by Code for blood.<sup>5</sup> Five cubic centimeter samples of the CSF were used. The extracts were assayed on the isolated, atropinized guinea pig ileum preparation suspended in Tyrode solution at 38° C. All results are expressed as histamine base in gamma per 100 cubic centimeters.

### SUBJECTS

The two groups of patients studied were from the neurological and neurosurgical services of the Royal Victoria Hospital and the Montreal Neurological Institute. The first group, comprising nineteen patients who presented no signs of meningeal irritation, served as controls. In all, routine examination of the CSF showed a cell count of 0 to 5 per cmm, total protein of 20 to 45 mg per cent, negative Wassermann, and the findings were considered normal.

The second group was made up of fifteen patients in whom abnormal CSF findings were observed and on which twenty-one histamine determinations were made. In twelve of these patients, the CSF fluid was obtained during the postoperative period. Six patients had the postoperative course complicated by an acute aseptic recurrent meningitis, a condition which follows intracranial surgery and was described by Finlayson and Penfield in 1941.<sup>6</sup> They observed that after the removal of a cerebral neoplasm or atrophic epileptogenic lesion, a blood clot is left behind which undergoes degeneration and liquefaction in the operative cavity. The breakdown products of the blood clot accumulate and are discharged into the subarachnoid space. Here they irritate its lining, resulting in an outpouring of leucocytes. This syndrome is characterized by repeated bouts of meningeal irritation which are associated with an increase in the number of leucocytes and protein values in a sterile spinal fluid. Patients 26 and 27 (see Table II) had their postoperative course complicated by a bacterial meningitis (*Bacillus coli* and hemolytic streptococci). A glioma of the third ventricle and a cerebellar cystic astrocytoma respectively were removed. The spinal fluid in three patients (26, 27, and 28) was withdrawn during the early postoperative period at which time the fluid showed a mild aseptic reaction as evidenced by an increase in the leucocyte count and protein value. This is a fairly

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common finding postoperatively and is usually of no consequence. The four nonoperated patients (31, 32, 33, and 34) presented abnormal CSF values resulting from their underlying intracranial pathology as shown in Table II.

TABLE I

PATIENT	SEX	DIAGNOSIS	CEREBROSPINAL FLUID			
			APPEARANCE	TOTAL PROTEIN (MG %)	HISTAMINE ( $\gamma/100$ c c)	
1 W S	M	Headache of undetermined origin	Clear and colorless	5	0.5	
2† L C	F	Cranio-cerebral injury subdural hematoma	Clear and colorless	44	0.8	
3 V D	M	Epilepsy, focal	Clear and colorless	32	0.8	
4† U H	M	Subdural hematoma	Clear and colorless	25	0.8	
5 F L	M	Anxiety neurosis	Clear and colorless	38	1.5	
6 L B	M	Mental defective	Clear and colorless	34	0.8	
7† B T	F	Meningeal fibroblastoma	Clear yellow	29	0.7	
8 R L	M	Epilepsy	Clear and colorless	22	1.5	
9 R K	M	Epilepsy	Clear and colorless	34	0.4	
10 P B	M	Epilepsy, focal	Clear and colorless	35	0.8	
11 M S	F	Epilepsy	Clear and colorless	31	0.8	
12 R D	F	Astrocytoma diffusum	Clear and colorless	16	0.2	
13 R O'D	M	Trigeminal neuralgia	Clear and colorless	25	3.0	
14 P P	M	Cranio-cerebral injury fractured skull	Clear and colorless	40	0.8	
15 E P	M	Cerebral atrophy	Clear and colorless	16	0.9	
16 W L	M	Epilepsy focal	Clear and colorless	28	0.6	
17 D D	M	Normal	Clear and colorless	-	0.7	
18 B	M	Normal	Clear and colorless	-	0.9	
19 W C	M	Normal	Clear and colorless	-	0.8	
					Average	0.97

The cell count in the spinal fluid ranged from 0 to 6 lymphocytes per cubic millimeter.

†The spinal fluid was taken during this patient's postoperative period.

## RESULTS

The values obtained in the group of control cases are shown in Table I. It will be seen that the average histamine content of the CSF is 0.97  $\gamma$  per 100 c.c., with variations of 0.2 to 3.0  $\gamma$  per 100 cubic centimeters. The highest value 3.0  $\gamma$  per 100 c.c. was found in the CSF of a patient with trigeminal neuralgia. It will be noted that all the CSF specimens from this group were clear and colorless with the exception of that from Patient 7.

In the second group of cases, twenty-one samples of CSF were obtained for analysis. The results are shown in Table II. These samples varied in appearance from colorless to xanthochromic and from clear to turbid. The total protein was increased in all specimens, the highest value being 854 mg per cent (Patient 20). Each sample moreover, had an increase in cell count with the exception of that from Patient 34, in which only the protein value was slightly elevated. The number of cells ranged from 0 to 11,000 per cubic millimeter. These were either wholly lymphocytic or polymorphonuclear or occasionally both. In a few specimens red blood cells were found. It will be observed that the average histamine content of the CSF in this group of patients was 1.43  $\gamma$  per 100 c.c. as compared with 0.97  $\gamma$  per 100 c.c. for the controls.



# OBSERVATIONS ON THE HISTAMINE CONTENT OF THE CEREBROSPINAL FLUID IN MAN

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IT IS generally agreed by most workers that 80 to 90 per cent of the blood histamine content is held within the white cell elements,<sup>1, 2</sup> but there is considerable controversy as to which of the white cells carries this bound or intracellular histamine. Some believe that the polymorphonuclear eosinophile is responsible,<sup>3</sup> whereas others consider that the platelets are mainly involved.<sup>4</sup>

Since cerebrospinal fluid may contain polymorphonuclear or lymphocytic cells as a result of meningeal irritation, it was thought that a study of the histamine content of such cerebrospinal fluid, as well as that of normal samples, might provide some evidence as to which type of cell is responsible for carrying histamine.

## METHODS

Cerebrospinal fluid collected by lumbar puncture in the case of normal subjects, or directly from the ventricle as well as by tap in the neurosurgical patients, was extracted for histamine by the method described by Code for blood.<sup>5</sup> Five cubic centimeter samples of the CSF were used. The extracts were assayed on the isolated, atropinized guinea pig ileum preparation suspended in Tyrode solution at 38° C. All results are expressed as histamine base in gamma per 100 cubic centimeters.

## SUBJECTS

The two groups of patients studied were from the neurological and neurosurgical services of the Royal Victoria Hospital and the Montreal Neurological Institute. The first group, comprising nineteen patients who presented no signs of meningeal irritation, served as controls. In all, routine examination of the CSF showed a cell count of 0 to 5 per cmm, total protein of 20 to 45 mg per cent, negative Wassermann, and the findings were considered normal.

The second group was made up of fifteen patients in whom abnormal CSF findings were observed and on which twenty-one histamine determinations were made. In twelve of these patients, the CSF fluid was obtained during the postoperative period. Six patients had the postoperative course complicated by an acute aseptic recurrent meningitis, a condition which follows intracranial surgery and was described by Finlayson and Penfield in 1941.<sup>6</sup> They observed that after the removal of a cerebral neoplasm or atrophic epileptogenic lesion, a blood clot is left behind which undergoes degeneration and liquefaction in the operative cavity. The breakdown products of the blood clot accumulate and are discharged into the subarachnoid space. Here they irritate its lining, resulting in an outpouring of leucocytes. This syndrome is characterized by repeated bouts of meningeal irritation which are associated with an increase in the number of leucocytes and protein values in a sterile spinal fluid. Patients 26 and 27 (see Table II) had their postoperative course complicated by a bacterial meningitis (*Bacillus coli* and hemolytic streptococci). A glioma of the third ventricle and a cerebellar cystic astrocytoma respectively were removed. The spinal fluid in three patients (26, 27, and 28) was withdrawn during the early postoperative period at which time the fluid showed a mild aseptic reaction as evidenced by an increase in the leucocyte count and protein value. This is a fairly

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The average histamine content of the CSF from the group with aseptic recurrent meningitis was 1.64  $\gamma$  per 100 cc, while the three specimens from the patients with bacterial meningitis averaged 1.87  $\gamma$  per 100 cubic centimeters. An average histamine content of 0.97  $\gamma$  per 100 cc of CSF was found in the group with early postoperative reactions, whereas only 0.87  $\gamma$  per 100 cc was found in the CSF of the patients who had not been operated on. The variations in these four subgroups correspond in general to the number of leucocytes present in the CSF and do not appear to be related to the amount of spinal fluid protein or the underlying pathology.

TABLE III

NUMBER OF CELLS IN CEREBROSPINAL FLUID PER CC	TYPE	HISTAMINE CONTENT OF CEREBROSPINAL FLUID ( $\gamma$ /100 cc)
11,000	I polymorph	3.0
9,000	I polymorph	1.6
6,000	I polymorph	2.0
2,100	Polymorphs	2.7
2,000	I polymorphs	3.0
1,500	I polymorph	0.8
1,015	Polymorphs	1.5
1,000	Polymorph	1.3
640	Polymorphs	0.5
200	Lymphocyte	1.0
200	Lymphocyte	2.0
120	Lymphocytes	2.0
83	Lymphocyte	0.8
40	Lymphocyte	2.0

In Table III the histamine values are arranged according to the descending order of cell counts. It will be observed that there is some correlation between the number of polymorphonuclear cells and the histamine content of the CSF but that this is far from constant. There seems to be little relationship between the histamine content and the lymphocyte count of the CSF although the histamine values are elevated.

## DISCUSSION

Blood plasma contains on the average 0.5  $\gamma$  histamine per 100 cc and it will be noted that this value is not dissimilar to that obtained for normal CSF. While the number of determinations in each group is small, it seems clear that an increase in the cell count of CSF is accompanied by an increase in its histamine content. From the results obtained, however, there does not appear to be much difference as to whether these cells are lymphocytes or polymorphonuclear in type. Studies on the histamine content of the blood of man have shown that for the most part highest values are found when immature myelogenous cells prevail such as in the myelogenous leucemias.<sup>1,2</sup> We have been unable to show that the eosinophile is per se the white cell responsible for carrying the blood histamine<sup>3</sup> since marked variations in the percentage of the eosinophiles in the blood of the same patient taken at different time intervals was not paralleled by similar changes in the blood histamine content.

It would appear that the elevated CSF histamine values observed in recurrent aseptic meningitis are not characteristic of this particular syndrome, but are merely a result of the white blood cells present in the spinal fluid, as evidenced by values obtained in other meningeal abnormalities. It is difficult to interpret the value of 3.0  $\gamma$  per 100 cc which was found in Patient 13 (Table I), since the cell count and protein values were within normal limits.

#### SUMMARY

1 The histamine content of the CSF was studied in a group of nineteen patients whose spinal fluid was normal and in a group of fifteen patients whose CSF was abnormal.

2 The average histamine content of normal CSF was found to be 0.97  $\gamma$  per 100 cc with variations of from 0.2 to 3.0  $\gamma$  per 100 cubic centimeters. Abnormal CSF was found to have an average histamine content of 1.43  $\gamma$  per 100 cc with variations of from 0.5 to 3.0  $\gamma$  per 100 cubic centimeters.

3 Some correlation between the number of cells present and the histamine content of CSF could be demonstrated. This was more marked for polymorphonuclears than for lymphocytes.

4 The underlying intracranial abnormality and increased CSF protein values did not appear to influence directly the histamine content of the spinal fluid.

We wish to thank Mrs. E. V. Harkness for carrying out the histamine assays.

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METABOLISM OF URIC ACID GLUTATHIONE AND NITROGEN, AND  
EXCRETION OF "11 OXYSTEROIDS" AND 17 KETOSTEROIDS  
DURING INDUCTION OF DIABETES IN MAN WITH  
PITUITARY ADRENOCORTICOTROPIC  
HORMONE

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AND ELIZABETH PINKHAM, B.S.

IN A PREVIOUS communication<sup>1</sup> it was reported that a typically diabetic state can be produced in man by the intramuscular administration of a purified preparation of adrenocorticotrophic hormone (A.C.T.H.). It was observed that loss of carbohydrate tolerance was not related to increased glycogenesis from protein. Severe negative nitrogen balance may accompany loss of carbohydrate tolerance induced by A.C.T.H. but the existence of one is not dependent upon the presence of the other.

It was reported further that a remarkably close correlation exists between loss of carbohydrate tolerance and the increased urinary excretion of uric acid which accompanies continued administration of A.C.T.H. It was suggested on the basis of a few determinations of blood glutathione done on one subject, that heightened intracellular production of uric acid and its intermediaries produced by A.C.T.H., together with a fall of the levels of glutathione and perhaps of other sulfhydryl bearing substances may impair functional activity of the beta cells of the islets of Langerhans.

The present report deals with experiments designed to explore further the relationship between loss of tolerance for carbohydrate, increased production of uric acid and changes in the levels of blood glutathione. Two normal men and a patient suffering from gout were used as the subjects of these studies. The procedure and chemical methods employed were the same as those described previously<sup>1</sup> with the exception that all determinations for urinary uric acid\* were performed according to the method of Buchanan, Block and Christman. Urinary "cortin" was determined by the method of Daughaday, Jaffe and Williams.<sup>13</sup>

The A.C.T.H.† used in this study was prepared‡ by a modification of the method of Sayers and co-workers.<sup>3</sup> The laboratory has supplied us with the properties of these materials (Table I). Because the diabetogenic potency of

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\*We are indebted to Dr. J. R. Mote, The Armour Laboratories, Chicago, Ill., for the purified adrenocorticotrophic hormone used in these studies.

†In the Armour Laboratories.

TABLE I PROPERTIES OF THE A C T H PREPARATIONS EMPLOYED

LOT	A C T H ACTIVITY (PER CENT OF ARMOUR STANDARD*)	PRESSOP ACTIVITY— ROOSTER B P (UNITS/MG)	OXYTOGIC ACTIVITY— GUINEA PIG UTERINE STIMP (UNITS/MG)	PPOLACTIN ACTIVITY— PIGEON COPP SAC (UNITS/MG)	GONADO TROPIC ACTIVITY— COLLIP (UNITS/MG)	SOLUBILITY
37 K G	34.8 ± 2	0.066	0.017	0.5	2.0	Soluble in saline
G 59703	15.8	0.0045	0.0036	1.0	1.5	Soluble in saline

\*The biologic activity of Armour A C T H Standard (L-1-4) is such that a single intravenous injection of 0.004 mg produces consistently a 20 to 30 per cent decrease in adrenal-ascorbic acid content of the hypophysectomized rat

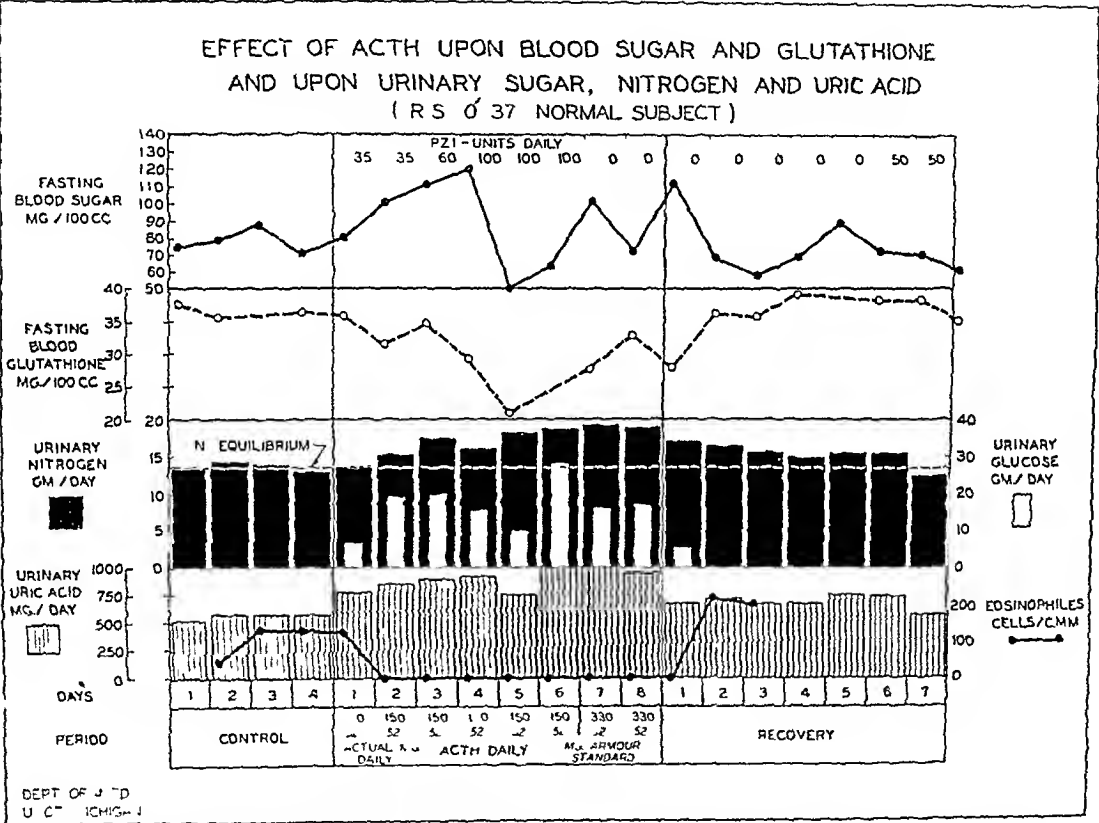


Fig 1—A C T H during the first six days was from Lot 37-K G During the last two days Lot G 59703 was employed

Lot G-59703 was found to be considerably less than that observed for Lot 37-K G (although equal potency for the two preparations was observed for other metabolic responses), material from each lot was administered in consecutive periods to each of the three subjects. Thus, Subject D E received material from Lot G-59703 for six days and from Lot 37-K G on the seventh and eighth days of his A C T H period. Subject R S received Lot 37-K G for six days and Lot G-59703 on the seventh and eighth days. Subject E S (gout) received Lot 37-K G for three days and Lot G-59703 on the fourth and

fifth days of A C T H administration. In this way the factor of individual variation in responsiveness to A C T H was minimized and any changes in blood glutathione, urinary uric acid and urinary "count" associated with the induction of glycosuria by the lot possessing the more potent diabetogenic property were controlled by the material which had but a mild diabetogenic effect.

### RESULTS

All of the results dealing with blood sugar, carbohydrate tolerance, blood glutathione, urinary sugar, nitrogen and uric acid are shown graphically on Figs 1 through 6.

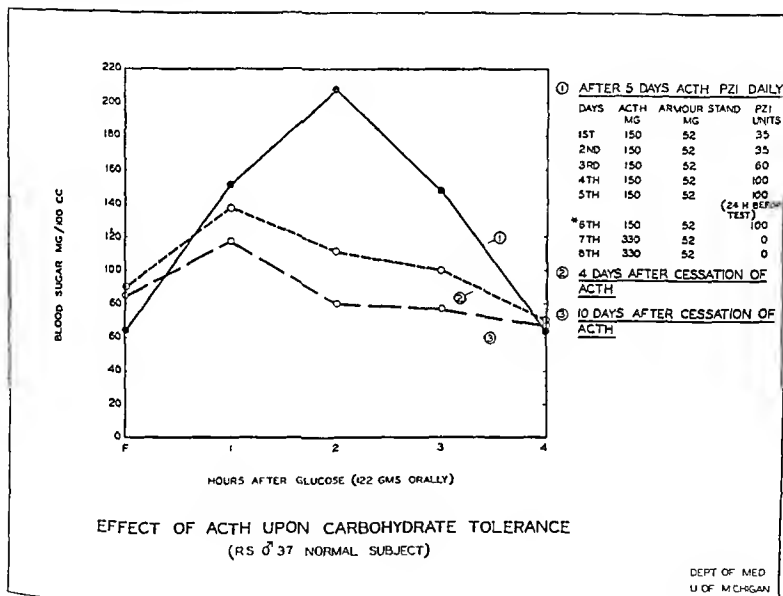


Fig 2—A C T H during the first six days was from Lot 37 K G. During the last two days Lot G 59703 was employed.

*Glycosuria and the Level of the Fasting Blood Sugar*—All three subjects developed glycosuria when given 52 mg (Armour Standard) per day of Lot 37 K G (Figs 1, 3 and 5). In Subject R S (Fig 1), 52 mg per day of Lot G 59703 maintained glycosuria initiated by Lot 37 K G but in Subject E S (Fig 5) the glycosuria disappeared when the switch was made. In Subject D L (Fig 3) no glycosuria occurred with 26 mg per day of Lot G 59703 but it did appear when the dose of this material was increased to 52 mg per

day A great increase of glycosuria occurred abruptly when 52 mg per day of Lot 37-K G were given on the seventh and eighth days It is clear that Lot 37-K G had the more intense diabetogenic effect

Maximal twenty-four hourly glycosuria for the three subjects was 117 grams (D E ), 138 grams (E S ), and 280 grams (R S ), the latter despite the administration of 100 units of protamine zinc insulin per day Glycosuria was not accompanied by polyuria Retention of sodium, chloride, and water<sup>4</sup> occurring simultaneously with the glycosuria tended to diminish urinary volume moderately

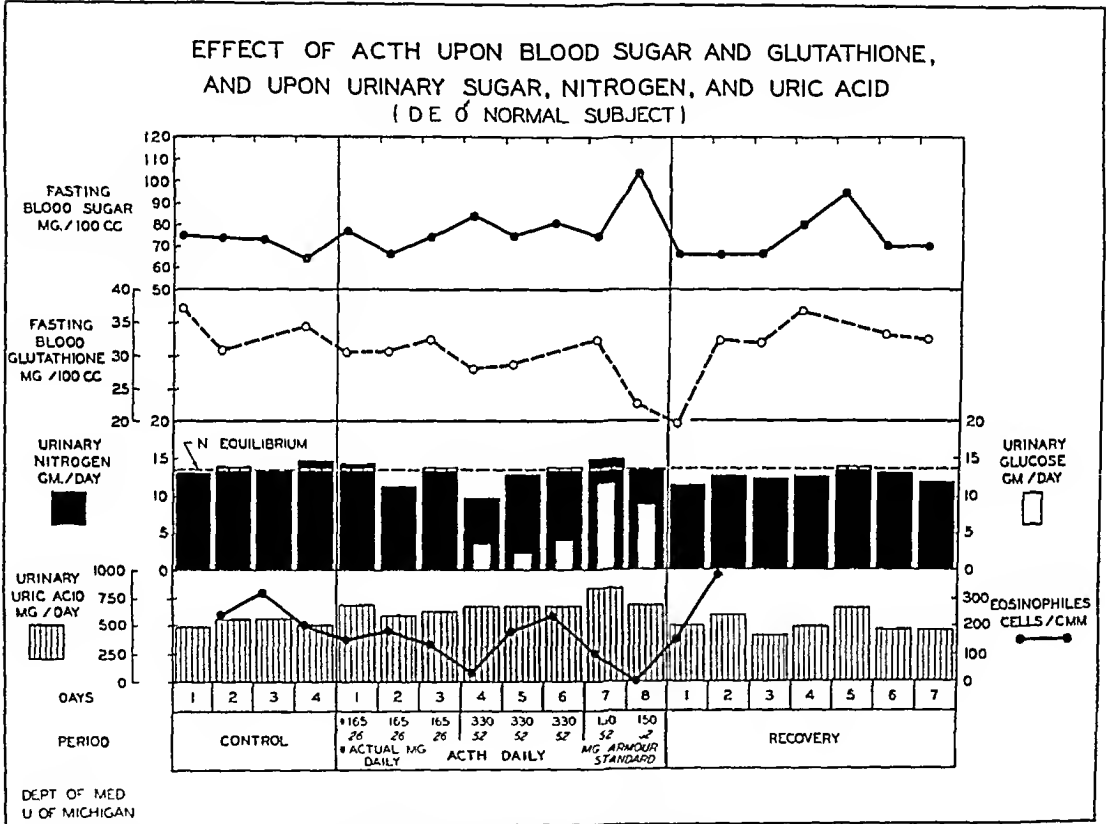


Fig 3—A C T H during the first six days was from Lot G-50703 During the last two days Lot 37-K G was employed

The maximal rise of the fasting blood sugar above the average base line value amounted to 47 per cent (D E ), 37 per cent (E S ), and 56 per cent (R S ), the latter during administration of protamine zinc insulin

*Carbohydrate Tolerance*—Subject R S (Fig 2) gave a typically diabetic response after five days of A C T H (Lot 37-K G) It should be noted that a total of 330 units of protamine zinc insulin had been administered during the same five day period and that it had failed to prevent the development of the diabetic tolerance curve even though it had finally halted the progressive rise of the fasting blood sugar level (Fig 1) Four days after cessation of

A C T H the curve indicated great improvement, and by the tenth post A C T H day recovery of carbohydrate tolerance was complete (Fig 2)

Subject D E, after five days of A C T H (Lot G 59703) showed a mild if significant, decrease in carbohydrate tolerance (Fig 4). On the seventh and eighth days of injection he received material from Lot 37 K G and the glycosuria increased abruptly along with a rise of the fasting blood sugar (Fig 3). Four days after cessation of A C T H, carbohydrate tolerance was still impaired but it was normal seven days later (Fig 4).

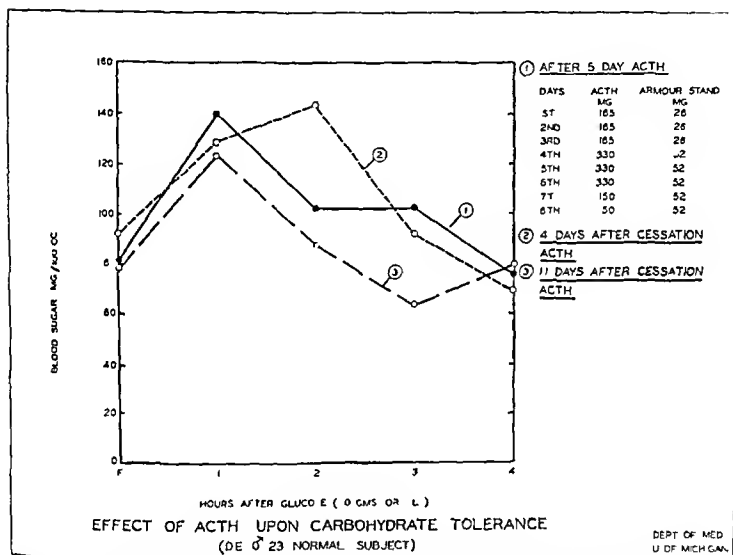


Fig 4—A C T H during the first six days was from Lot G 59703. During the last two days Lot 37 K G was employed.

Subject D S, chosen for this study because he had a pre-existing defect in uric acid metabolism (gout), reacted to A C T H in much the same way as did the normal subjects. Fig 6 shows that after twenty-four hours of A C T H (a total of 41 mg of Lot 37 K G) an intense loss of carbohydrate tolerance occurred. The initial control curve had been obtained two days previously. The next test done after two more days of injection of the same lot of A C T H indicated this subject's tendency to escape from the diabetogenic effect of the hormone. The glycosuria produced by Lot 37 K G disappeared entirely when Lot G 59703 was used (Fig 5). Normal tolerance for carbohydrate had returned by the sixth day following cessation of A C T H injections.

*Uric Acid Metabolism and the Level of Blood Glutathione*—The close relationship between increased urinary excretion of uric acid induced by



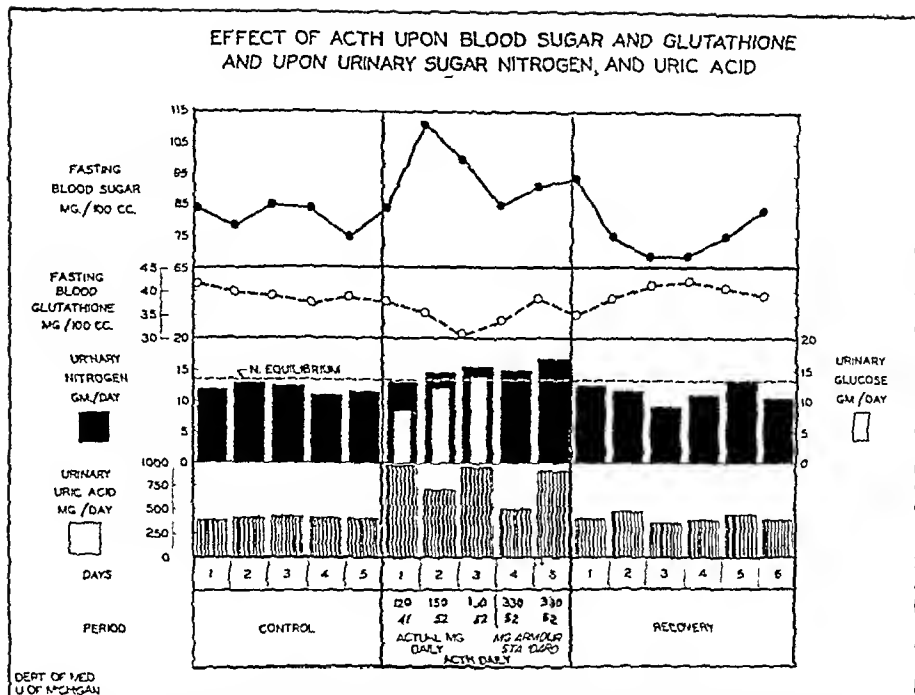


Fig 5—A C T H during the first three days was from Lot 37-K G During the last two days Lot G-59703 was employed

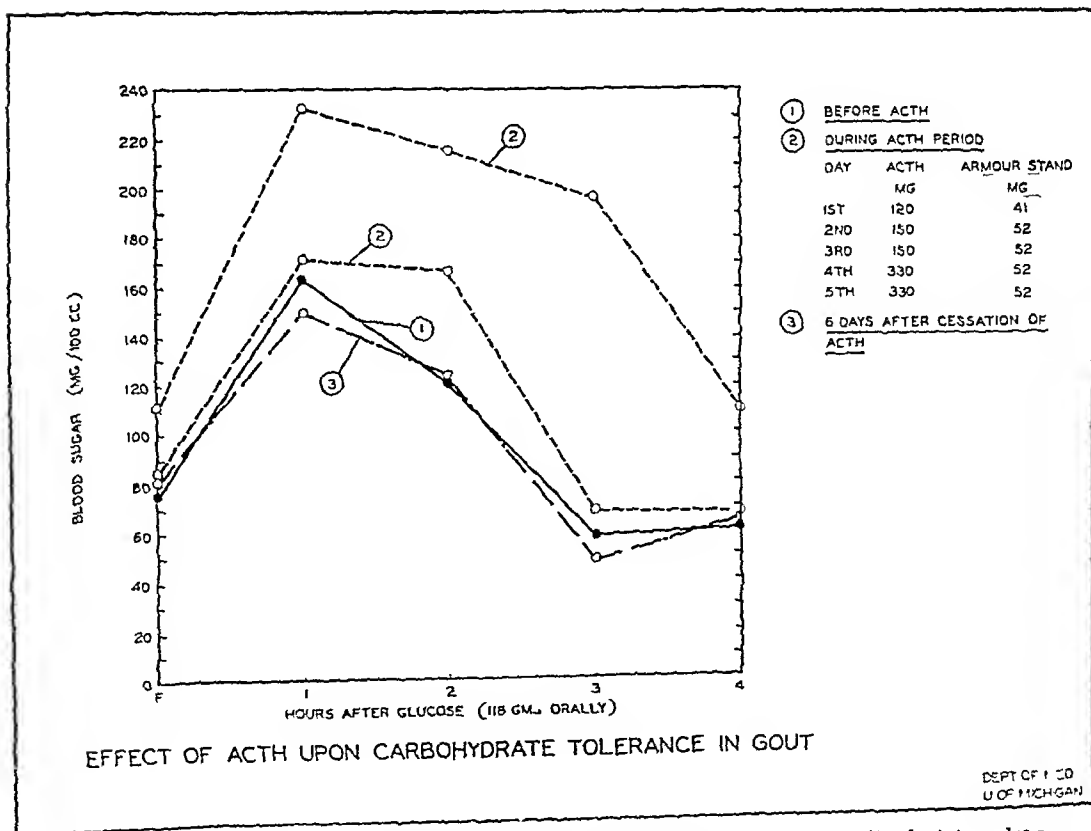


Fig 6—A C T H during the first three days was from Lot 37-K G During the last two days Lot G-59703 was employed

A C T H, and the development and persistence of glycosuria is observed again in this study (Figs. 1, 3, and 5) as it was in the previous one.<sup>1</sup> Note however, that the induction of glycosuria is related not only to increased production of uric acid but also to a concurrent fall of the level of blood glutathione, that the lower the glutathione in relation to a given level of heightened purine metabolism, the more intense is the glycosuria, and that a major difference between the highly diabetogenic preparation (Lot 37 K G) and less diabetogenic one lies in the capacity of the former to produce a sharper decrease in the level of blood glutathione. Subject D T (Fig. 3) demonstrates all three of these phenomena. With 26 mg per day of Lot G 59703, a rise in the excretion of uric acid occurred. This was not associated with a fall of the level of blood glutathione and glycosuria did not occur. When the dose of the same material was doubled a small amount of glycosuria occurred. Urinary uric acid did not change significantly but blood glutathione was mildly depressed. When 52 mg per day of Lot 37 K G were used blood glutathione fell precipitously and glycosuria increased sharply. There occurred too a mild increase of urinary uric acid. Upon discontinuation of A C T H a sharp rise of blood glutathione occurred.

Table II gives an index of the relative potencies of these two preparations of A C T H with respect to an effect unrelated to carbohydrate metabolism, namely, the increased excretion of 17 ketosteroids induced by the materials. Subject D T produced the same increase of 17 ketosteroids on 52 mg per day of Lot G 59703 as he did on 52 mg per day of Lot 37 K G. Yet the effect of the latter preparation on carbohydrate metabolism and upon blood glutathione (Fig. 3) was much more intense than that of the former.

The results of Subject H S (Fig. 1) leave no doubt that blood glutathione falls significantly in an individual who responds to A C T H with marked glycosuria. The administration of insulin did not prevent the fall of blood glutathione even though it was finally able to depress the level of the fasting blood sugar and to reduce the glycosuria. When on the last two days of A C T H the change was made from Lot 37 K G to Lot G 59703 glycosuria diminished even though no insulin was given. Blood glutathione rose. Urinary uric acid, urinary nitrogen, circulating eosinophiles and 17 ketosteroids (Table II) remained at levels which indicated continued activity of A C T H at least with respect to those indices. Thus it appeared that Lot 37 K G contained more of something which tended to depress blood glutathione than did Lot G 59703.

Subject D S (Fig. 5) demonstrated a small but definite fall of the level of blood glutathione. While Subjects R S and D E each showed a maximal fall of blood glutathione (below the average base line value) of 45 per cent that for E S was only 18 per cent. This may have been a factor in the ability of E S to recover tolerance for carbohydrate despite continued administration of Lot 37 K G (Fig. 6). It is interesting to note again that 52 mg per day of Lot G 59703 were roughly equivalent to 52 mg per day of Lot 37 K G in its ability to stimulate increased excretion of 17 ketosteroids (Table II).

TABLE II DAILY EXCRETION OF URINARY STEROIDS IN RESPONSE TO TWO PREPARATIONS OF ACTH

PERIOD	DAYS	SUBJECT R S (NORMAL)				SUBJECT D E (NORMAL)				SUBJECT E S (GOU)			
		ACTH (MG/DAY)	CORTIN (MG/DAY)	17 KETO STEROIDS (MG/DAY)	ACTH (MG/DAY)	CORTIN (MG/DAY)	17 KETO STEROIDS (MG/DAY)	ACTH (MG/DAY)	CORTIN (MG/DAY)	17 KETO STEROIDS (MG/DAY)	ACTH (MG/DAY)	CORTIN (MG/DAY)	17 KETO STEROIDS (MG/DAY)
Control	1	0	—	151	0	—	129	0	0.9	116	0	0.9	116
	2	0	1.2	181	0	0.9	126	0	1.7	107	0	1.7	107
	3	0	0.9	162	0	0.6	134	0	1.1	138	0	1.1	138
	4	0	0.6	178	0	0.7	100	0	1.1	126	0	1.1	126
	5	0	1.0	180	0	0.7	126	0	1.0	94	0	1.0	94
ACTH	1	52*	3.1	276	26†	1.8	123	41*	1.4	224	41*	1.4	224
	2	52*	2.9	455	26†	1.6	140	52*	1.6	396	52*	1.6	396
	3	52*	2.9	618	26†	1.3	192	52*	2.6	452	52*	2.6	452
	4	52*	2.2	484	52†	1.4	201	52†	4.2	455	52†	4.2	455
	5	52*	4.4	568	52†	1.4	223	52†	2.6	449	52†	2.6	449
	6	52*	3.3	504	52†	2.0	271	—	—	—	—	—	—
	7	52†	1.0	143	52*	2.6	275	—	—	—	—	—	—
	8	52†	3.8	507	52*	8.5	275	—	—	—	—	—	—
Recovery	1	0	1.5	212	0	5.1	113	0	1.8	152	0	1.8	152
	2	0	1.5	189	0	5.9	94	0	1.1	62	0	1.1	62
	3	0	1.8	168	0	1.3	106	0	1.2	53	0	1.2	53
	4	0	1.4	172	0	1.3	100	0	1.2	69	0	1.2	69
	5	0	1.4	110	0	0.9	130	0	1.6	91	0	1.6	91
	6	0	1.2	169	0	1.7	169	0	1.8	116	0	1.8	116
	7	0	1.6	129	0	5.1	189	—	—	—	—	—	—

\* (Armour Standard) — Lot 37-K G

† (Armour Standard) — Lot G-59703

and in its capacity to promote negative nitrogen balance (Fig 5). Mention should be made of the fact that A C T II produced no greater urinary excretion of uric acid in a patient with gout than it did in normal individuals.

*Protein Metabolism*—As noted previously<sup>1</sup> one finds again complete lack of correlation between protein metabolism and the occurrence of glycosuria. In Subject R S (Fig 1) negative nitrogen balance persisted for six days following cessation of A C T II, but glycosuria disappeared on the second post-injection day. Subject D E (Fig 2) developed no negative nitrogen balance as the result of A C T II. Nevertheless glycosuria appeared. It became evident first during a phase of nitrogen retention. This too has been noted before.<sup>1</sup> Subject E S (Fig 5) developed negative nitrogen balance and glycosuria under the influence of Lot 37 K G. When he was switched to Lot G 59703 negative nitrogen balance persisted but glycosuria disappeared promptly.

*Urinary Excretion of "Cortin" and of 17 Ketosteroids*—In the light of the metabolic changes presented it is of interest to examine in detail the results obtained from daily analyses of the urine for excretory steroids with particular reference to "cortin" (Table II). A great rise in the excretion of 17 ketosteroids was observed during the A C T II period in all three subjects. The maximal response observed for each subject was obtained and maintained with either preparation of A C T II administered at the same dose level. It is significant that regardless of which preparation had been maintaining the elevated excretion of 17 ketosteroids during the last two days of A C T II cessation of injections resulted during the next twenty-four hour period in a sharp fall of 17 ketosteroid excretion to levels approaching the base line values. This means that when excessive stimulation by A C T II of androgenic corticoid production ceases, excessive production of such steroids diminishes rapidly. It means, too, that urinary excretion of the metabolic end products (17 ketosteroids) is rapid in relation to the time of production of their precursors.

In contrast complete excretion of the cortin-like steroids appears to be characterized by a lag of about forty-eight hours after their production and after their physiologic effect has been observed. Subject D E (Table II) is seen to have shown his greatest rise of urinary "cortin" on the second day of injections of Lot 37 K G. A very high level of "cortin" excretion continued for two days after the injections were stopped. On the basis of these urinary values alone one might assume that time is required before the adrenal cortex responds to the stimulus, and that the cortex continues to produce excessive amounts of "cortin" for several days after the stimulus stops. A glance at Fig 3, however, is sufficient to destroy this interpretation. From a metabolic point of view, there is evidence of great increase of activity of "11 oxysteroids" only during the two days that Lot 37 K G was injected, and the most intense metabolic changes occurred during the first day of injection of this material (Day 7). During the first two recovery days (when "cortin" excretion was very high) all evidence of increased physiologic activity of cortin-like steroids had disappeared.

Similar interpretations cannot be made with equal assurance in the other two subjects because in them Lot 37 K G was given initially and they received

Lot G-59703 only during the last two days of injection (a time during which increased excretion of "cortin" would have been expected without injections, had they reacted similarly to Subject D E) Nevertheless, there is some evidence for existence of this same phenomenon In Subject E S (Table II) urinary "cortin" excretion reached its peak value during the fourth day of A C T H (three days of Lot 37-K G and one day of Lot G-59703) If urinary "cortin" were reflecting endogenous production of that material on the day of its excretion, one would expect the maximal metabolic effect to have been observed on the fourth day It is significant, however (Fig 5), that on the fourth day metabolic indices of "11-oxy steroid" activity had fallen sharply, particularly with reference to carbohydrate metabolism It seems most likely that the high urinary excretion of "cortin" on the fourth day of A C T H was due in part, at least, to excretion of metabolic end products of corticosteroids which had been produced earlier and which had exerted metabolic effects during the initial three days of injections The data on Subject R S (Table II and Fig 1) are not easily interpreted in this regard because this subject had received large amounts of insulin during the first six days of A C T H and none during the last two days That there existed excessive "11-oxy steroid" activity during the last two days of injections is obvious Whether it was as intense as during the preceding days is difficult to evaluate All things considered, the proposition seems justified that the metabolic end products of the 11 and 11-17 oxy steroids appear in the urine much later in relation to their time of production than do the excretory products of the androgenic corticosteroids

In connection with the variations in total nitrogen metabolism which are observed in different individuals exposed to the *same* adrenal cortical stimulus, it is of interest to examine the relative quantities of the two major types of excretory steroids which appear in the urine Subjects B S and E S developed negative nitrogen balance Total increases in both 17-ketosteroid and "cortin" excretion were of the same order of magnitude and proportionality Subject D E, who demonstrated no loss of body protein, showed much *lower* total excretions of 17-ketosteroids During the first six days of A C T H the proportionality of 17-ketosteroids to "cortin" was similar to that observed in the other two subjects But in the latter part of the A C T H period, when "cortin" excretion rose abruptly, unaccompanied by a rise of 17-ketosteroid excretion, no significant change in nitrogen equilibrium occurred (Fig 3 and Table II) It seems probable that over-all nitrogen balance depends upon the relative amounts of protein-anabolic and protein-catabolic steroids produced in response to an adrenal cortical stimulus but that the total quantity of "cortin" excreted gives no index of the amount of the protein catabolic steroid which contributed to the excretion of the total quantity of "11-oxy steroids"

#### DISCUSSION

Our previous report<sup>1</sup> and the present one indicate that glycosuria, increased levels of the fasting blood sugar, and glucose tolerance curves characteristic of the diabetic state can be produced in normal people by means of

the administration of suitable amounts of pituitary preparations, purified so as to contain mainly the adrenocorticotrophic hormone. It seems likely that in response to A.C.T.H. the elaboration of excessive amounts of certain of the adrenal corticosteroids (of the 11 and 11-17 oxygenated types) plays an important part in the initiation of the diabetic state. It is unlikely that the small amounts of known contaminants present in these preparations are capable per se of producing diabetes mellitus. Not ruled out are the possibilities (1) that one of these known contaminants potentiates the diabetogenic activity of the cortical steroids which are produced (2) that an unknown factor is present in the purified A.C.T.H. which increases the diabetogenic potentiality of excessive production of the so-called "S" type of corticosteroids or (3) that glycosuria is dependent upon excessive production of a specific 11 or 11-17 oxysteroid in that preparations of A.C.T.H. vary in their capacities to provoke increased production of that particular steroid. One of these possibilities is suggested by the experiments shown in Figs. 1, 3, and 5 in which Lot 37 K G was found to exert a much greater diabetogenic effect than Lot G 59703 when both preparations were alternated in the same three subjects. It seems significant too that in other respects the two preparations produced similar effects. In this connection it is evident that Lot 37 K G depressed the level of blood glutathione much more than did Lot G 59703. The possibility then that another factor is required in addition to increased "S hormone" activity in order that a marked diabetogenic effect be produced in the intact organism cannot yet be discarded. That there may exist more than one pituitary adrenocorticotrophic hormone must also be considered.

From the metabolic point of view the close association of impaired carbohydrate tolerance, increased uric acid excretion and depressed levels of blood glutathione is intriguing. The induction of alloxan diabetes in animals is associated with an abrupt fall of the blood glutathione to 50 per cent or more below the base line value.<sup>6</sup> Animals that resist the diabetogenic effect of alloxan show a much smaller, if any depression of blood glutathione.<sup>6</sup> Suitable administration of large amounts of glutathione or of other sulfhydryl bearing compounds protects against the diabetogenic effect of alloxan.<sup>7</sup> Whether or not alloxan or other related compounds which selectively injure beta cells are normal intermediaries of purine metabolism they are chemically related to uric acid. Compounds of this family which are diabetogenic exert this effect only in the presence of a subnormal level of blood reduced glutathione. It has been reported recently by Griffith<sup>8</sup> that hyperglycemia can be produced in rabbits by intraperitoneal administration of uric acid providing that the level of blood glutathione is first lowered by means of a diet deficient in cystine and in methionine. These findings appear to be related to ours.

That an increased excretion of uric acid occurs in normal human beings given A.C.T.H. or adrenal corticosteroids of the 11 or 11-17 oxygenated types is now well known.<sup>9</sup> Whether the fall of blood glutathione which we have observed with A.C.T.H. is the result of an increased endogenous production of

alloxan or alloxan-like intermediaries associated with heightened puime metabolism (and thus an indirect effect of "11-oxy steroid" activity), or whether this fall is produced by an extraneous factor cannot be said at this time. In any case the data indicate (Fig 3) that in the presence of increased urinary excretion of uric acid a sharp fall of the level of blood glutathione is associated with an increase of glycosuria. Similarly, even in the presence of increased puime metabolism a rise of blood glutathione is associated with a decrease of glycosuria (Fig 1).

It is conceivable that in the presence of both lowered intracellular concentrations of reduced glutathione and heightened intracellular concentrations of puime metabolites, enzymatic production of insulin by the beta cell is impaired. This does not imply necrosis of beta cells, such as occurs following a large dose of alloxan during which the exposure of these cells is intense but transitory. In fact, the effective period after administration of alloxan is so transitory that exposure *must* be intense in order that any effect be demonstrable. However, when the cell is given a much smaller degree of the same qualitative exposure over a relatively prolonged period of time, it is logical to believe that the initially demonstrable abnormality will be functional rather than anatomical. It is probable, too, that the very function for which this cell exists (production of insulin) is the most important factor which makes it so selectively susceptible to the conditions mentioned. While this concept, with respect to production of a temporary diabetic state in normal human beings by means of the materials utilized in these experiments, is not firmly established it is believed that it constitutes a beginning which warrants further intensive investigation.

That there is involved, too, an extrapancreatic diabetogenic effect seems likely. In the experiment in which insulin was administered (Fig 1) the subject showed a greater resistance to insulin than is usually observed in a diabetic patient who exhibits a similar degree of reduction of carbohydrate tolerance. It could be argued that A C T H-induced diabetes is one which is based *entirely* upon a decreased effectiveness of endogenous insulin in the tissues and that the beta cells may be producing actually more than the normal quantity of insulin in response to "tissue requirements", that under these circumstances, fatigue of the beta cells will soon supervene, as evidenced by retrogressive cytologic changes and decreased secretory activity. It will be agreed, however, that if A C T H were still being administered at this time, resistance to exogenous insulin still would be evident. It is clear, then, that resistance to exogenous insulin gives no information relative to the total production of insulin by the pancreas. As judged by the amount of functioning pancreatic tissue which is needed in man, following partial pancreatectomy, to prevent the appearance of diabetes, A C T H-induced diabetes is compatible with a very significant decrease of the capacity of the beta cells to make insulin. It is to be recalled, too, that partially depancreatized animals, even though not diabetic, are much more susceptible to the diabetogenic effect of A P E (anterior pituitary extract) than are animals with pancreas intact.

The assumption that a decreased functional capacity of the beta cells occurs during A C T H administration (as a result of the metabolic aberration described) is easily reconcilable with the classical experimental results of Best,<sup>10</sup> Houssay,<sup>11</sup> and Lukens<sup>1</sup> and their respective associates. Best and co-workers<sup>10</sup> showed the existence of a *decreased content of pancreatic insulin* of dogs by the third day of A P E administration. On the basis of associated retrogressive changes in the beta cells it was concluded with justification, that the phenomenon was the result of extrapancreatic, hyperglycemic overstimulation of the beta cells. That this mechanism is an essential part of the evolution of beta cell degeneration goes without saying. Houssay and co-workers<sup>11</sup> observed a decreased *insulin producing capacity* of the pancreases of A P E treated dogs after three daily injections of the material but were unable to duplicate these findings in dogs given no A P E but given glucose in sufficient amount to maintain a hyperglycemia of the same degree and for as long as that observed in the A P E treated animals. It was shown too that as the functional reserve of the pancreas is diminished (by increasing degrees of partial pancreatectomy), increased sensitivity to the diabetogenic effect of A P E occurs. Dohan and Lukens<sup>1</sup> have demonstrated that hyperglycemia *per se if sufficiently intense and if sufficiently prolonged* is capable of inducing degenerative changes in the beta cells. The extreme resistance of normal beta cells to degeneration by means of hyperglycemia alone is noteworthy in their experiments. This is far different from the experience of all who have worked with A P E. In the latter case, both functional and retrogressive cytologic changes of the beta cells are demonstrable in three to four days during which time the hyperglycemia is much less intense than that which was found necessary in the glucose experiments of Dohan and Lukens. We suggest that the difference may be explainable on the basis of the increased vulnerability to hyperglycemia of beta cells which have been damaged functionally by the metabolic upheaval of purine and glutathione metabolism induced by A C T H.

Whatever may be the mechanism or combination of mechanisms which initiates impaired carbohydrate tolerance and hyperglycemia when purified A C T H is administered, it is reasonably certain that in highly responsive individuals such as Subject R S continued administration of the material would eventually result in permanent diabetes. Obviously, such an experiment is not justifiable. That there is great individual variation in the susceptibility of carbohydrate metabolism to injury by the measure used is clear. The variation may reside in differences in the capacities of normal individuals to maintain a reasonably normal level of reduced glutathione and of other sulfhydryl bearing compounds under the conditions imposed. This factor appears to be a determining one with respect to the diabetogenic potency of administered alloxan in animals. One could speculate that this factor may be involved in the well known familial susceptibility to diabetes mellitus.

A final comment is required concerning the changes in protein metabolism which were observed in the subjects of this investigation. Upon administration of A C T H the exhibition by a given subject of negative nitrogen



balance, nitrogen equilibrium, or positive nitrogen balance is dependent very likely upon the relative proportions of androgenic (protein anabolic) and of certain of the 11 and 11-17 oxygenated corticosteroids (protein catabolic or antianabolic) that are released in response to the stimulus<sup>4</sup>. But glycosuria occurred in all of our subjects regardless of the status of nitrogen metabolism. While glycosuria and loss of carbohydrate tolerance are more intense in those who demonstrate negative nitrogen balance, the latter is not a necessary accompaniment. It is believed that the emphasis, rather than being upon excessive glycconeogenesis from protein, should be upon the fact that A C T H causes an over-all reduction of the capacity of the body to utilize normally those elements which enter into the "carbohydrate pool," regardless of their source. Loss of body protein and decreased carbohydrate tolerance are metabolic effects probably produced via the same types of corticosteroids. In different individuals, however, these effects may vary independently of each other indicating (1) that each is not dependent for its existence upon the other and (2) that such variations may be manifestations of individual differences in end organ responses to similar stimuli, or manifestations of individual differences in the production of specific steroids belonging to the 11 and 11-17-oxy steroid family.

#### SUMMARY

A state of metabolism similar in all respects to clinical diabetes mellitus has been produced in normal human beings by the administration of a purified preparation of adrenocorticotrophic hormone. It seems evident from the results that permanent diabetes mellitus in man could be produced with this material were caution not exercised with respect to duration of administration and dosage employed. The diabetes evidenced during such treatment was found to be relatively resistant to exogenous insulin. This fact does not indicate necessarily that resistance to endogenous insulin is the mechanism by which loss of carbohydrate tolerance is affected by A C T H.

Analysis of the metabolic data discloses a consistent correlation between loss of tolerance for carbohydrate, increased endogenous purine metabolism, and depressed levels of blood glutathione. It is suggested that A C T H may affect changes in the composition of intracellular fluid which include (1) increased concentrations of intermediaries of purine metabolism and (2) decreased concentrations of sulfhydryl bearing compounds. It is suggested, further, that since the beta cells of the islets of Langerhans are known to be more sensitive to injury by the aforementioned intracellular alterations (probably by virtue of their insulin-producing function) than are any other cells of the body, a functional impairment of these cells occurs which increases their vulnerability to hyperglycemia.

Negative nitrogen balance is not a necessary accompaniment of glycosuria produced by A C T H. Although greater loss of carbohydrate tolerance is observed in subjects who lose body protein, the two metabolic phenomena are not mutually dependent. Specific steroids of the 11 or 11-17 oxygenated types may be responsible for each phenomenon.

Some of the data suggest that there may exist more than one pituitary adrenocorticotrophic hormone

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# INTENTIONAL ISOIMMUNIZATIONS AGAINST THE ANTIGEN D (Rh<sub>0</sub>)

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NATURALLY occurring anti-Rh antibodies either do not exist or are exceedingly rare. Those produced by isoimmunization in pregnancy are frequently of low titer and many times contain a mixture of antibodies, all factors making them an unreliable and sometimes undesirable source of this highly sought after laboratory reagent.

With the report by Hill, Haberman and Oiozco<sup>1</sup> on preparation of anti-Rh sera by injection of Rh-positive cells into previously immunized Rh-negative women, a method became available to increase the supply of this valuable reagent\*. Previously it had been shown that experimental sera produced in various animals not only have the disadvantage of small quantity and low titer but exhibit a certain lack of specificity, particularly when tested against cord cells or cells from newborn infants.

The next important contribution toward production of specific and potent sera was made by Wiener and Gordon<sup>2</sup> who carried out intentional immunizations of normal Rh-negative men by injection of Rh-positive cells of a known specificity. Their injection schedule called for two intravenous injections of 4 cc each of a 50 per cent red cell suspension at four-month intervals, followed by bleeding of the donors approximately ten days after the last injection. By this method they sensitized five of nine individuals but obtained only two sera sufficiently potent for laboratory purposes. In an addendum they report a yield of five useful sera from six injected individuals immunized over a period of one year with injections at six-week intervals.

While the studies described below were in progress, Van Loghem<sup>3</sup> reported the production of anti-C (1h') and anti-E (1h'') sera by means of artificial immunization of volunteer donors.

The purposes of our investigation were to determine the types of antibodies produced as well as the order of their appearance, the incidence of individuals who could be immunized by the method used, and the minimum quantity of red blood cells required to produce immunization. An additional important consideration was our endeavor to obtain a supply of potent anti-Rh serum.

## METHOD

Ten white Rh negative volunteers who had never received transfusions or intramuscular injections of blood as far as could be ascertained were selected for this experiment. Each of them received an initial intravenous injection of a 50 per cent cell suspension of the type

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\*At the International Hematological and Rh Conference (Dallas Texas 1946) L. Diamond reported unpublished experiments with intentional immunizations dating back to 1942. By 1946 he had treated over sixty-five subjects with 75 per cent success.

O Dec (Rh)<sup>+</sup> On the basis of a 47 per cent hematocrit this was calculated to contain approximately 117 cc of packed cells. All subsequent injections were administered at six week intervals and group O type  $\frac{DcF}{DcE}$  (Rh homozygous) cells were used for these and all other injections. Each dose comprised a packed cell volume of 1 cubic centimeter. Before the initial injection and at the time of each subsequent injection, sera were obtained from all ten individuals and tested for the presence of antibodies by means of the saline agglutination technique, the 20 per cent bovine albumin, and the Coombs antihuman precipitin technique,<sup>4</sup> using the injected cells and a cell of the type DCE as antigens. As soon as any sensitization could be detected the injection schedule for the particular individual was changed to ten day intervals in order to speed up the process of antibody formation. All sera containing antibodies were tested for their specificity with appropriate cells.

## RESULTS

The first antibody response occurred simultaneously in three individuals after the third injection. The cell volume injected up to that time comprised a total of 317 cc each. After the fourth injection, four subjects responded with antibody formation, and after the fifth and sixth injections seven and nine

TABLE I

NUMBER OF IMMUNEFESPONSES	NUMBER OF INJECTIONS	APPROXIMATE CELL VOLUME INJECTED (CC)
0	1	117
0	2	217
3	3	317
4	4	417
7	5	517
0	6	617

sensitizations respectively could be detected. The maximum total volume of cells injected into any one volunteer was 617 cubic centimeters. These results are summarized in Table I.

As shown in Table II, the three individuals who produced antibodies after only three injections responded with formation of saline agglutinins to a titer of about 1:10. The reactions obtained in 20 per cent bovine albumin were found

TABLE II

SUBJECT	TITER		
	SALINE AGGLUTINATION	20 PER CENT BOVINE ALBUMIN	COOMBS REAGENT
Ma	10	10	160
Bu	10	10	160
Me	<10	<10	<10

to be within approximately the same range, whereas the Coombs reagent in two out of the three sera demonstrated antibody titers which far exceeded those obtained by either the saline or albumin techniques.

All four subjects who required only three or four stimulations produced initially saline agglutinins which in the course of further injections showed a

This suspension was prepared by withdrawing 30 ml of venous blood from the donor and mixing it with an equal amount of 3.6 per cent sodium citrate in a sterile bottle. The resulting suspension was thoroughly mixed by shaking and 5 ml were used for each injection.

definitive rise and fall in titer, even to a level of complete disappearance.\* At the same time the reactions elicited in 20 per cent bovine albumin media increased in titer consistently. The titer of antibodies detected by means of the Coombs technique exceeded the titers in bovine albumin in two of the four cases whereas identical results were observed in the other two. The above-mentioned titration difference amounted to between three and four double dilutions. Typical samples of these findings are shown in Table III.

TABLE III

NUMBER OF INJECTIONS	TITR					
	SUBJECT MA			SUBJECT ME		
	SALINE AGGLU TITERS	20 PER CENT BOVINE ALBUMIN	COOMBS REAGENT	SALINE AGGLU TITERS	20 PER CENT BOVINE ALBUMIN	COOMBS REAGENT
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	8	8	128	<8	<8	<8
4	8	128	512	32	32	32
5	256	256	1024	256	256	256
6	16	256	2048	0	512	512

The remaining five cases which required five or more injections for production of antibodies failed to show saline agglutinations. The titration values in bovine albumin and those obtained with the Coombs reagent ranged from 1/2 to 1/64, but in no case did the Coombs technique detect antibodies of significantly greater strength than those demonstrated in albumin media. One subject (C B) who responded after the fifth injection was found to have lost all detectable antibodies after a subsequent injection. Because the original titer was low these results were seriously questioned, but on re-examination of the old specimen

TABLE IV

NUMBER OF INJECTIONS	TITR								
	SUBJECT F S			SUBJECT K			SUBJECT C B		
	SALINE AGGLU TITERS	20 PER CENT BOVINE ALBUMIN	COOMBS REAGENT	SALINE AGGLU TITERS	20 PER CENT BOVINE ALBUMIN	COOMBS REAGENT	SALINE AGGLU TITERS	20 PER CENT BOVINE ALBUMIN	COOMBS REAGENT
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	8	16	0	4	4	0	2	2
6	0	32	64	0	8	8	0	0	0

preserved at icebox temperature the antibodies found previously could still be detected. One of the ten cases failed altogether to show any antibody response after six administrations of six-weekly injections amounting to approximately 6 cc of packed cells. Typical examples of the late immune response are shown in Table IV.

Three of the donors having sera with maximum titers of 2048, 512, and 64 respectively were retested eight weeks after their last injection. None showed

\*The same observation was made by Levine and Wigod in cases of isoimmunization in pregnancy. (Personal communication.)

diminution in titer or qualitative change in antibodies. The same sera were again retitrated after six months of storage at +7° C and the results were identical or within one double dilution which is considered to be within the limits of the experimental error.

With reference to the specificity of the antibodies produced in the nine volunteers, eight were found to correspond completely to anti D (Rh<sub>0</sub>). One individual (Mr.), however, was found to have produced anti C (rh') and anti D (Rh<sub>0</sub>) antibodies of equal titer and quality. Because of this unexpected result the serum was submitted to Dr. Philip Levine who was able to confirm our observation. Consequently, because it was suspected that the cells used for our immunization were actually Dce (Rh<sub>1</sub>) and not Dce (Rh<sub>0</sub>) or DeE (Rh<sub>1</sub>), they were subjected to a battery of anti C sera in our laboratory as well as in Dr. Levine's. All tests failed to disclose the presence of a C antigen. Because of the experience of Van Loghem, who by injection of cells presumably of type dCe (rh), but actually D Ce obtained in anti D + C (Rh'<sub>0</sub>) serum, the cells used in our experiments were submitted to Dr. R. R. Race in London for detection of an intermediate C antigen. Dr. Race reported the absence of C, C<sup>u</sup>, C<sup>w</sup> or c<sup>w</sup>, thus leading us to believe that the volunteer must necessarily have received other Rh positive blood at a previous occasion. Further search into his history by close questioning and conference with his mother failed to reveal any previous incidence of intravenous or intramuscular injection of blood. It is of interest to note that this same individual was the only one who reported a reaction\* after every injection including the first one and was the only one who during the series of injections developed infectious hepatitis.

#### DISCUSSION AND SUMMARY

With the method presented, it was possible to immunize nine of ten human subjects using six intravenous injections to a total of about 6 ml of cells. Five of the individual sera produced were sufficiently potent to be used as laboratory reagents.

The immune response between those who required only three or four injections and those requiring more seemed to differ significantly. The absence of saline agglutinins in the late immunizations is rather striking. As observed in some sera there was a marked difference between reactions obtained in albumin media and those obtained with the Coombs reagent. Although it is well known that this reagent is more sensitive than any other method used for the detection of sensitization it is difficult to understand why this difference in titer should appear only in some sera and not in all. One possible explanation may be the presence of a third order antibody as previously postulated by Hill and Harberman.<sup>5</sup> It is of interest to note that this discrepancy in titer was observed only in those volunteers who were immunized with relative ease.

As regards the stability of the antibodies produced they were found to be at least as stable or more stable than those occurring in cases of isoimmunization in pregnancy.

\* Pain in eyeballs and lumbar region, slight elevation of temperature and chills

The disappearance of antibodies after a subsequent injection of the antigen, as occurred in one case, is compatible with the phenomenon of negative phase of immunization which was recently observed to occur even during natural immunizations against Rh<sup>6</sup>

The presence of anti-C in addition to anti-D in the serum Ma cannot be explained satisfactorily. Although nonspecific antibody production is known to occur, as for instance described for the M and N system by Landsteiner and Levine,<sup>7</sup> this is usually observed in low titers. The fact that the nonspecific antibody observed in our case was of high titer and equal to the specific one makes this explanation less likely. In attempting to explain the phenomenon observed, the following possibilities should be kept in mind. First, the history may have been incorrect and our volunteer possibly received an injection of foreign blood as a child. This appears most likely because of the reaction reported by him even after the first injection. Second, it is remotely possible that the cells used for injection contained a hitherto undetected variation of the antigen C, capable of producing anti-C antibodies. Third, one should consider that other antigenic stimuli, even the attack of homologous serum hepatitis, may have been responsible for the nonspecific antibodies observed.

In comparing the minimum amounts of cells necessary to produce immunization against C (1h') and E (1h'') in the series of Van Loghem<sup>3</sup> and those found necessary in our experiments, the following results can be obtained (Table V)

TABLE V

ANTIGEN INJECTED	D	C	E
Minimum amount of cells necessary to immunize (e c)	3 17	6 5	13 0

From these findings it appears that with the decrease in antigenicity of the cells the quantity of antigen must be doubled. Whether these results are significant or accidental can be established only by a larger series.

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## LABORATORY METHODS

### DETERMINATION OF PHENOL IN BIOLOGIC MATERIAL

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THERE are a number of color reactions for phenol available some of which lend themselves readily for the determination of phenolic substances in biologic material. Tests for phenol were reviewed by Gibbs<sup>1</sup> in 1926 and their applications to biology by Deichmann and Schaefer in 1942.

For biologic uses the most commonly employed methods are the molybdenum blue method of John and Coerlitz<sup>2</sup> and the azo dye method introduced by Thiers and Benedict.<sup>3</sup> The latter method is distinctly preferable because of its far greater sensitivity and specificity. Its main disadvantage is the lability of the diazo reagent which must be prepared fresh daily. To obviate this disadvantage it was attempted to utilize one of the commercially available stabilized diazo salts which under suitable conditions have good keeping properties. Fifteen such compounds were tested and, although several of them proved to be usable diazotized 5 nitro 2 aminoanisole\* was selected as the reagent of choice because of the intense orange red shade it gives on coupling with phenol. This shade is quite stable in the presence of 4 to 6 per cent alcohol whereas in a purely aqueous medium it gradually fades. Alcohol in concentrations in excess of 7 per cent inhibits full development of color. With phenolic substances other than phenol itself the optimal concentration of alcohol may be higher or lower also the shade and intensity of the color of the azo dye may be different. Certain substances such as salicylic acid and p substituted phenols give pale shades. Table I shows the molar efficiency (i.e., the ratio of

molar concentration of phenol  
molar concentration of substance in question,

giving the same colorimetric readings) of the method in the case of various phenolic substances. In addition a number of nonphenolic compounds considered likely to give color are also listed.

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Red B Salt National Aniline Naphthanol Diazo Red B DuPont Fast Red Salt V Ciba



TABLE I SENSITIVITY OF AZO DYE METHOD TO VARIOUS SUBSTANCES

SUBSTANCE	[MOLAR EFFICIENCY]	REMARKS
Phenolic compounds		
Phenol	1	
Salicylic acid	0.035	
Tyrosine	0.09	
Thymol	1.17	17% alcohol, purple
o Cl phenol	1.13	
Guaiacol	1.32	
o Cresol	1.34	
p Cresol	0.18	
Resorcinol	0.77	
Alpha naphthol	1.51	40% alcohol, blue
Nonphenolic compounds		
Acetone	0.00013	
Arginine	0.005	
Histidine	0.015	
Tryptophane	Less than 0.001	

As shown, the molar efficiency for substances of nonphenolic nature is so low as to rule them out as sources of error

#### METHOD

*Reagents*—1 Saturated solution of borax in 15 per cent alcohol To 1,000 ml of 15 per cent alcohol add about 35 Gm of powdered borax ( $\text{Na}_2\text{B}_4\text{O}_{10} \cdot 10\text{H}_2\text{O}$ ) and shake repeatedly Some of the borax will remain undissolved This reagent is stable

2 Diazo reagent Dissolve 0.25 Gm of Red B Salt (or any of the other brands mentioned) in 100 ml of ice cold water and filter Add 1 ml of 5 per cent  $\text{H}_2\text{SO}_4$  This solution will keep in the ice box for over two weeks

*Standardization of Curve*—Prepare a stock solution of phenol (reagent grade, purity  $\pm 99$  per cent) containing 1 mg of phenol in 10 milliliters Make a series of dilutions by pipetting 0.5, 1, 2, 3, and 5 ml of the stock solution into 100 ml volumetric flasks and filling them up to the mark with water Measure 5 ml samples of each (covering the range of 2.5 to 25  $\mu\text{g}$  of phenol per sample) into colorimetric test tubes, use the same amount of water as a blank Add to each tube 1 ml of water and 4 ml of borax solution Invert tubes twice Add 0.5 ml of diazo reagent Invert tubes twice Read between three and sixty minutes after mixing, using a 490  $\text{m}\mu$  (blue green) filter Plot data on semilogarithmic graph paper A straight line should be obtained

*Determination of Phenol in Unknown*—Unknown (urine, serum, and so forth), 1 ml, water, 5 ml, borax solution, 4 ml, diazo reagent, 0.5 milliliter Read against blank as before In case the specimen is not colorless, two blanks must be used A, specimen replaced by water, B, diazo reagent replaced by water Both unknown and the blanks are read against pure water, and the sum of values for A and B is deducted from the value for the unknown If, owing to large amounts of phenol present, the shade is too dark for reading, both unknown and blank are diluted to a convenient larger volume (e.g., 25 or 50 ml) with 4 per cent alcohol, and the values are multiplied by a corresponding factor The amount of diazo reagent given is sufficient up to 120  $\mu\text{g}$  of phenol For special purposes, the technique may be modified For instance, in the case of a Folin-Wu filtrate, take a 5 ml sample, add 1 ml of 0.3N NaOH (to neutralize the acid), 4 ml of borax, and 0.5 ml of diazo reagent

#### RESULTS

The present method was used for the determination of phenol in serum (ten cases), in Folin-Wu filtrates of serum (five cases), in histamine-stimulated gastric juice (191 cases), and in urine (forty-nine cases) The readings on serum were invariably higher (range, 17 to 32  $\mu\text{g}/\text{ml}$ ) than in the corresponding Folin-Wu filtrates (range, 7 to 10.4  $\mu\text{g}/\text{ml}$ ) because phenolic groups in

proteins also react. The range in gastric juice was 1.4 to 56  $\mu\text{g}/\text{ml}$ , in urine, 0.9 to 44  $\mu\text{g}/\text{milliliter}$ . All urine containing more than 25  $\mu\text{g}$  of phenol were positive with Ehrlich's diazo reaction.

#### OTHER APPLICATIONS OF THE PHENOL REACTION

The reaction described can be used for the determination of phosphatase activity, both in the acid and alkaline range with the advantage that the step of deproteinization becomes unnecessary. The introduction of a new substrate (phenyl benzoate) permits the determination of lipase (esterase) activity in a highly accurate, simple and inexpensive way.

TABLE II. EFFECT OF BORATE ON HYDROLYSIS OF PHOSPHORIC ESTERS

(Enzyme Solution of purified extract of dog intestine 1:500 dilution, 1 ml. Substrates 0.005M in 0.05M buffer of pH 9. Mg 0.005M. Activity expressed in  $\mu\text{g}$  of P liberated in 1 hour Temp, 37° C.)

SUBSTRATE	ACETATE BUFFER	BORATE BUFFER
Glycerophosphate	102	57
Phenylphosphate	93	96

**Phosphatases**—The substrate is King and Armstrong's<sup>5</sup> di Na phenyl phosphate. The buffer in the acid range is acetate while in the alkaline range it is borate. The latter is a cheap and efficient buffer at the optimum range of alkaline phosphatase in case of phenylphosphate as a substrate (around pH 9.8).<sup>6</sup> In preliminary experiments it was shown that borate does not inhibit the hydrolysis of phenylphosphate while it strongly inhibits that of glycerol phosphate (Table II).

**Reagents**—1. Substrate: di Na phenylphosphate approximately 0.05M. Use a good grade chemical, low in phenol or, if such is not available extract about 1.1 Gm. of di Na phenylphosphate with about 100 ml. of hot alcohol, dry and dissolve it in 100 ml. of distilled water.

2. Acetate buffer, 0.05M, pH 5. Dissolve 4.75 Gm. of Na acetate and 0.9 ml. of acetic acid in 1,000 ml. of water.

3. Borate buffer, 0.1M, pH 9 to 9.8. Dissolve 9.5 Gm. of borax and 30 to 35 ml. of 4 per cent NaOH in 1,000 ml. of water.

4. Twenty per cent solution of magnesium chloride.

**Determination of phosphatase activity**—A. Alkaline phosphatase. Buffered substrate (borate buffer, 90 ml. substrate 10 ml. Mg solution, 1 ml. mixture will keep in the refrigerator for several months), 5 ml., serum dilution 1:25 (1 milliliter. Blank buffered substrate without serum. Incubate tubes at 37° C. for one hour. Place tubes in ice cold water. Add to blank 1 ml. of serum dilution. Determine phenol as described.

B. Acid phosphatase. Buffered substrate acetate buffer, 90 ml., substrate 10 milliliters. This mixture will undergo slow hydrolysis even in the refrigerator and should not be kept for longer than two weeks. Procedure otherwise is identical with the previous one.

There are a number of arbitrary units of phosphatase activity described in literature four of which are being widely used (Bodansky,<sup>7</sup> King Armstrong,<sup>5</sup> Huggins and Talalay's<sup>8</sup> phenolphthalein, and Bessey and co workers<sup>9</sup> antrophenol units). It is suggested in order to facilitate direct comparison of results obtained with various methods that the activities of all hydrolytic enzymes be expressed in terms of micromoles of ester linkages split in one hour by 1 ml. (or 1 Gm.) of biologic material preferably under optimal conditions or in some cases under the special conditions of the experiment re-

gardless of the compound actually determined colorimetrically or by any other means, and regardless of the actual length of time of incubation. Table III gives a comparison between the various units of alkaline phosphatase activity, the determinations having been performed with human serum exactly according to the authors' specifications. It should be remarked, however, that neither the Bodansky nor the King-Armstrong technique utilizes optimal conditions. Both omit the addition of Mg, and for this reason the Mg concentration is suboptimal ( $10^{-4}$  M or less, instead of  $10^{-3}$  to  $10^{-2}$  M), moreover, in the

TABLE III EQUIVALENTS OF UNITS OF ALKALINE PHOSPHATASE ACTIVITY

UNIT	DEFINITION	EQUIVALENTS
Bodansky	1 mg of P/h/100 ml	1.24
King-Armstrong	1 mg of phenol/30 min/100 ml	2.60
Nitrophenol	1 mM of nitrophenol/h/1,000 ml	0.69
New method	1 $\mu$ M of phenol/h/ml	1

case of the latter one, the pH of the buffered substrate is below the optimum (9.3 to 9.35 instead of 9.8). The ratios may be significantly different in the case of alkaline phosphatases other than that of human serum (unpublished data). No direct comparison between the phenolphthalein unit and the other units is possible because the relationship between the amount of phenolphthalein liberated and the amount of enzyme is not rectilinear.

The phosphatase activities of fifty-one unselected human sera were determined by the new method. The range of normal values at alkaline pH was from 2.3 to 6.6  $\mu$ M/milliliter. Four sera from patients with obstructive jaundice read 9.2, 12.6, 13.4, and 16.2  $\mu$ M/milliliter respectively. The range of values at acid pH was from 0.64 to 1.48  $\mu$ M/milliliter.

*Esterase (Lipase)*—Techniques utilizing the Warburg apparatus are obviously not suitable for the purposes of a clinical laboratory, those based on titration of acid liberated<sup>10, 11</sup> are not sensitive enough, the results of the stalagmometric method<sup>12</sup> do not lend themselves to a strict quantitative evaluation. The first colorimetric method sufficiently sensitive and simple to be used in the clinical laboratory is the p-nitrophenol ester method of Huggins and Lapides.<sup>13</sup> Its disadvantage is the lability of the substrate, necessitating the frequent preparation of fresh solutions and the running of the test at a nonoptimal pH.

The method to be described utilizes phenyl benzoate as a substrate. This ester is crystalline, available practically phenol-free,\* and fairly stable both in alcoholic and aqueous solutions. Fatty acid esters (acetate, propionate and butyrate) of phenol were found to contain significant amounts of free phenol which it was impossible to remove. Phenyl benzoate is only very slightly soluble in water (about 40 mg/l at 23° C) but as will be shown, the saturated solution is entirely satisfactory for the measurement of esterase activity within wide limits.

Phenyl benzoate is readily hydrolyzed by both lipase and esterase as shown in Table IV. The rates of hydrolysis of methyl butyrate and of Tween 20 were determined by the following adaptations of the author's methods for choline

\*Eastman No. 632

esterases<sup>14</sup> there was a replacement of substrates (methyl butyrate, 0.01 M Tween 20, 0.25 per cent), in addition, instead of using prostigmine, enzymatic activity was stopped by immersing the tubes in ice cold water. To the blanks the enzyme was added just before titration or turbidimetric reading. Gelatin was omitted from the CaCl<sub>2</sub> solution because it caused a turbidity with Tween 20. Hydrolysis of olive oil was determined by titration in 80 per cent alcohol.

TABLE IV RATES OF HYDROLYSIS OF VARIOUS SUBSTRATES BY LIPASE AND LESTPASE  
(Enzymes: 1:10 homogenates of guinea pig pancreas and liver and human serum. Activities expressed in  $\mu$ M of acid liberated by 1 ml in 1 hour.)

SUBSTRATE	LESTPASE (LIVER)	LIPASE (PANCREAS)	SERUM
Olive oil	None	165	None
Tween 20	46	116	8
Methyl butyrate	600	190	45
Phenyl benzoate	440	120	41

The dependence of the rate of hydrolysis on pH is shown in Table V. The optimum is between 6.0 and 6.5 which value is considerably lower than in the case of most other substrates (around pH 8).

TABLE V EFFECT OF pH ON RATE OF HYDROLYSIS OF PHENYL BENZOATE BY PANCREATIC LIPASE  
(Enzyme: Armour's pancreas powder 1% suspension diluted 1:50. Buffer: tris(hydroxy methyl) aminomethane maleate<sup>14</sup>)

pH	5.0	5.5	6.0	6.5	7.0	7.5	8.0
$\mu$ g of phenol/h	11	13.5	15.5	15	13	8.5	5.3

Hydrolysis is proportional to time for at least two hours provided not more than 30 per cent of the substrate (equivalent to 28  $\mu$ g of phenol in 1.5 ml sample) is used up (Table VI).

TABLE VI COURSE OF HYDROLYSIS IN TIME  
(Enzyme: human serum diluted 1:150)

TIME (MIN)	10	20	30	40	50	60	80	120
$\mu$ g of phenol	1.8	3.9	6.0	7.8	9.7	11.6	17.4	22.5

Provided not more than 30 per cent of the substrate is split in the course of the experiment, there is a linear relationship *within wide limits* between the amount of enzyme and the rate of hydrolysis (Table VII).

TABLE VII EFFECT OF ENZYME CONCENTRATIONS  
(Enzyme: as in Table V)

1 Enzyme dilution	500	250	200	100	50	25	20	16	13	10
2 Relative amounts of enzyme	2	4	5	10	20	40	50	63	75	100
3 $\mu$ g of phenol/h	0.6	1.8	2.2	4.3	8.4	16.0	21.2	25.4	29.4	31.5
4 $\mu$ g of phenol/relative amounts of enzyme	0.30	0.45	0.44	0.43	0.42	0.41	0.42	0.40	0.39	0.32

## METHOD

*Reagents*—1 Phenyl benzoate stock solution Dissolve 2 Gm of phenyl benzoate in 100 ml of methanol This solution will keep in the refrigerator indefinitely

2 Phosphate buffer, 0.1 M, pH 6.3 Dissolve 5.35 Gm of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and 7.0 Gm of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  in 1,000 ml of water

3 Buffered substrate into 500 ml of buffer blow 1 ml of substrate stock solution Stir Keep in the ice box The buffered substrate will undergo very slow hydrolysis, producing slightly colored blanks, but will remain usable for about two weeks Shake before use because some of the ester will precipitate in the cold

*Determination of esterase activity*—To 5 ml of prewarmed buffered substrate add 1 ml of enzyme (serum dilution 1:200, gastric juice 1:25, and so on) Procedure is otherwise identical with that for phosphatases

## RESULTS

The esterase activity of fifty-one human sera and of 242 gastric juices was determined The range in the sera was between 14.5 and 41.2  $\mu\text{M}/\text{milliliter}$  in forty-eight cases out of fifty-one Three cases had activities of 52, 47.5, and 54  $\mu\text{M}/\text{milliliter}$ , respectively The first two were cases of diabetes, and the third one was a psychiatric case In three cases parallel determinations of serum esterase activity by Huggins and Lapidus' p-nitrophenol propionate (PNPP) method and the new method were done It was found that 1 PNPP unit was equal to 0.3  $\mu\text{M}/\text{milliliter}$  according to the new method

The range of concentration of enzyme in gastric juices corresponded to 1.2 and 200  $\mu\text{M}/\text{milliliter}$ , 220 out of 242 cases having an activity lower than 40  $\mu\text{M}$  Gastric juices containing gross bile were especially numerous among cases with very high activities, indicating regurgitated intestinal and pancreatic juice as the possible source of the excess enzyme On the other hand, many juices containing gross bile showed low activities Table VIII shows the statistical distribution of esterase activity in gastric juices

TABLE VIII DISTRIBUTION OF ESTERASE ACTIVITY IN GASTRIC JUICES (242 CASES)

RANGE ( $\mu\text{M}/\text{ml}$ )	1-2.5	5-10	10-20	20-30	30-40	40-50	50-70	70-100	OVER 100
Number of cases	7	57	101	40	14	2	3	8	10
Gross bile	2	17	18	6	4	None	1	6	6

There is a marked irregularity in the shape of the frequency distribution curve between 30 and 70 micromoles For this reason, the cases showing activities over 70  $\mu\text{M}$  were analyzed separately in respect to diagnosis Nine patients had no demonstrable gastric lesions, five of them had normal acid values, and four were anaerid Of the remaining nine, two had carcinoma of the stomach, six had duodenal ulcers treated by x-ray irradiation (five anaerid, one normacid), and in one the diagnosis was atrophic gastritis with achlorhydria As far as the relationship between acid values in general and esterase

activity is concerned, Fig 1 shows a scattergram of eighty two cases selected at random from all ranges of acidity and esterase activity. Except for the fact that very high activities are almost invariably associated with low acid values or with anacidity there is no apparent correlation between acidity and esterase activity of gastric juice.

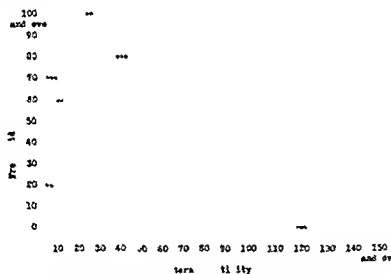


Fig 1—Relationship between free acid values and esterase activity

#### SUMMARY

A new and simple azo dye method for the determination of phenol phosphatases, and esterase in biologic material is described. Range of phosphatase and esterase values as determined with the new method is given.

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# A SIMPLE METHOD FOR THE DETERMINATION OF FECAL FAT AND FATTY ACIDS

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**I**N CLINICAL diagnosis of sprue,<sup>1</sup> celiac syndrome,<sup>2</sup> and cystic fibrosis, fecal fat determinations are often of value. The most commonly used methods are those of Kaye<sup>3</sup> and Tidwell and Holt.<sup>4</sup> The Tidwell and Holt method is time consuming, requiring 200 ml of solvent which must be evaporated to dryness on a steam bath. The Kaye method is even more tedious and requires eleven hours of extraction.

In the new method which we present here a result may be obtained within two and one-half hours after the stool is dry. This procedure is particularly applicable to routine hospital determinations in that forty determinations may easily be carried out in one working day.

We have found that stool may be dried to constant weight at 60° C overnight. An advantage of drying at 60° C is that no objectionable odors are evolved. At 100° C some pyrolysis takes place as indicated by the acrid odors given off. Drying at 60° C may therefore be done in an electric oven without the use of a hood. By homogenizing the stool and taking approximately a 2 Gm sample which is spread on the bottom of a 50 ml beaker or Petri dish, approximately forty samples may be dried in a standard electric incubator at one time. Where the original sample is small, as for infants, the stool is collected in shallow, wide-mouth jars which are placed directly in the 60° oven.

The dried stool is pulverized and samples are weighed into test tubes. To the test tube is added dilute HCl to free the fatty acids from the soaps and a petroleum ether-diethyl ether mixture to extract the lipids. One drop of ethyl alcohol is also added. This serves to prevent the mixture from emulsifying when shaken. The extraction is now accomplished by shaking in a standard shaking machine on which has been mounted a special carrier designed to accommodate large numbers of tubes. This carrier has been previously described.<sup>5</sup> The tubes are then centrifuged and the ether mixture is aspirated off. This process is repeated. The combined extracts are evaporated to dryness and weighed. Studies have shown that by these two extractions 98 to 99 per cent of the fat is extracted. Further extractions are therefore unnecessary for routine determinations.

If free fatty acid determinations are desired, alcohol is added to the extracted lipid and the solution is titrated with alkali. Thus by this method both total fat and fatty acids may be determined at the same time. Recoveries

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were made up by dissolving one part of stearic acid in two parts of molten lard, 185 mg of this mixture were added to 500 mg of dried stool. Table I summarizes the results of ten such recoveries.

TABLE I RECOVERY OF FAT AND FATTY ACID ADDED TO TEN SAMPLES OF DRIED STOOL

A	WEIGHT OF DRIED STOOL SAMPLE (MG)	WEIGHT OF TOTAL FAT EXTRACTED (MG)	FAT ADDED (MG)	FAT RECOVERED (MEAN) (MG)	AVERAGE DEVIATION FROM MEAN (%)
	500	133	185	185	±0.40
B	MEAN TITRATION OF FAT EXTRACTED FROM STOOL (0.1N NaOH)	MEAN TITRATION OF FAT ADDED (185 MG)	MEAN TITRATION OF FAT RECOVERED	AVERAGE DEVIATION FROM MEAN TITRATION	
	2.49 ml	1.79 ml	1.78 ml	±0.03 ml	

Table II shows typical results obtained in normal adults and children.

TABLE II PER CENT FAT IN STOOLS OF NORMAL ADULTS AND CHILDREN

CASE	AGE (YR)	WEIGHT OF FAT RECOVERED FROM 500 MG OF STOOL (MG)	PER CENT FAT IN DRY STOOL	CASE	AGE (YR)	WEIGHT OF FAT RECOVERED FROM 500 MG OF STOOL (MG)	PER CENT FAT IN DRY STOOL
1	2	48	9.6	10	5	99	19.8
2	40	76	15.2	11	1	68	12.6
3	36	84	16.8	12	2	98	19.6
4	1	99	19.8	13	52	94	18.8
5	53	128	25.6	14	21	64	12.8
6	20	29	5.8	15	50	93	18.6
7	5	111	22.2	16	5	104	20.8
8	35	56	11.2	17	34	47	9.4
9	45	32	6.4	18	25	33	6.6

#### REAGENTS

1 *Hydrochloric Acid (1N)*—Made up by diluting the concentrated acid (12N) to twelve times its volume with water.

2 *Petroleum Ether Ether Mixture 1:1*—Made by mixing equal quantities of redistilled petroleum ether (boiling point 70 to 90°) and redistilled diethyl ether.

3 *Ethyl Alcohol 95 Per Cent (Redistilled)*

4 *Sodium Hydroxide 0.1N*—This must be standardized daily against 0.1N standard sulfuric acid.

5 *Standard Fat for Recovery*—To 8 Gm of lard melted at 100° C were added 4 Gm of stearic acid. The mix was stirred until homogeneous and allowed to cool. The solid fatty mixture was dissolved in benzene and dried over CaCl overnight. The CaCl was filtered off and the benzene was evaporated off under vacuum. The residue was brought to constant weight in a vacuum desiccator.



## PROCEDURE

The total stool or approximately 2 Gm of the homogenized stool are dried in a beaker by placing it in an electric oven at 60° C overnight. Duplicate 500 mg samples are weighed into clean, dry 15 ml test tubes with either ground glass or Tygon stoppers. To each tube is added the following

5 ml 1N HCl

5 ml petroleum ether-ether mixture

1 drop 95 per cent ethyl alcohol

The tubes are carefully stoppered and shaken for twenty-five minutes in a shaking machine.

The Tygon stoppers are constructed by sealing both ends of a short piece of glass tubing (8 mm outside diameter, 1.5 cm in length). The sealed tube is inserted into a piece of Tygon tubing (outside diameter 1.5 cm, length 1.7 cm). These stoppers are then extracted with petroleum ether to remove any possibility of fatty materials being present. This should be repeated each time the stoppers are used.

After the tubes have been shaken, they are centrifuged at 2,500 revolutions per minute for fifteen minutes. The contents of the tube should then appear as in the following description. The bottom of the tube contains a sediment. Above this sediment is the aqueous layer and above this is most often found a layer of nonfat material in the form of a button. In the event that there is an emulsion formed at the interface of the aqueous and ether layer, add a few more drops of alcohol, shake thoroughly, and recentrifuge. Above the button of nonfat material is found the layer of ether mixture. The formation of the button facilitates the removal of the ether layer.

The ether mixture layer is now aspirated off into a weighed beaker, care being taken to avoid contamination of the ether layer with the aqueous layer. Five milliliters of the ether mixture are again added to the tube. The tube is placed again in the shaking machine and centrifuged as before. The ether from the second extraction is added to that of the first and the mixture is evaporated to dryness on a steam bath. The beaker containing the residue from the drying of the ether is now brought to constant weight in a vacuum desiccator and weighed. A blank is run in similar manner without added stool. The blank should be negligible. If more than 0.2 mg of residue is obtained the solvents need to be redistilled.

If free fatty acid determinations are desired, 10 ml of ethyl alcohol are added to the beaker and it is heated to just below the boiling point of the alcohol. This dissolves the fats and the solution is titrated with 0.1N NaOH while still hot. A blank consisting of 10 ml of alcohol is titrated at the same time. The indicator used for both titrations is a 1 per cent solution of phenolphthalein in alcohol. If the equipment is available, electrometric titration may be substituted.

## CALCULATION

1 Weight of beaker and fat - weight of beaker = Weight of fat

2  $\frac{\text{Weight of fat}}{\text{Dry weight of stool sample}} \times 100 = \text{Per cent fat in stool}$

(Titration of fat - titration of blank)

3  $\frac{\times \text{normality} \times 268 \times 100}{\text{Weight of fat in milligrams}} = \text{Per cent fatty acids in fat}$

In the third equation the number 268 is derived from the average molecular weight of fatty acids assumed by Tidwell and Holt <sup>4</sup>

## DISCUSSION

The method described requires no special equipment other than a means of shaking large numbers of test tubes simultaneously. In this manner determination of stool fat can be used with greater frequency as a diagnostic aid even in laboratories possessing limited equipment. Further, large numbers of determinations may be done simultaneously without the necessity of handling cumbersome equipment. No separator funnels or Soxhlet extractors need be used.

From Table I it can be seen that the results as obtained on recoveries indicate an accuracy in the order of the more tedious methods.

Table II lists results on normal adults and children. These results are comparable to those previously reported on normal individuals <sup>3</sup>

The method describes the determination of total fatty acids and total fat. No attempt to fractionate neutral soaps was made for it has been shown that the amount of free fatty acid is compared with neutral soaps varies with the method of drying the stool <sup>5</sup>. Kaye by a mild method of drying was able to get a higher percentage of free fatty acid for less was converted to calcium soaps than by more severe heating. Furthermore the ratio of sodium salts to fatty acids in stool is a function of the pH as would be expected from the basic equation

$$\text{pH} = \text{pK} + \log \frac{\text{Sodium salt of fatty acid}}{\text{Fatty acid}}$$

Thus, all the information required can be obtained from determinations of total fat, fatty acids, and pH. In our procedure the fatty acids are freed from combination with the alkali metals by shaking with the acid during the extraction.

This method has been in use in our laboratories for more than a year. During that time it has shown itself to be practicable and easily learned by the average technician.

## SUMMARY

1 A convenient method is described for the determination of fatty acids and fats in stool.

2 This method is simple, requiring no special equipment other than a shaking machine

3 The extraction is carried out in test tubes

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## SIMPLIFIED EQUIPMENT FOR DETERMINATION OF UROBILINOGEN IN URINE AND STOOL

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NUMEROUS observers<sup>1,2</sup> have in recent years stressed the usefulness of measurements of urinary and fecal urobilinogen in differential diagnosis and in following the course of certain diseases. Watson and associates<sup>3</sup> have described a simplified method for determination of urobilinogen which is quantitated by using a comparator block with pontacyl dye standards. This method has found extensive use but verbal reports indicate that many laboratories have not employed the procedure because of difficulties in preparing comparator blocks with materials at hand. The purpose of this paper is to describe the use of (1) a commercially available comparator block and (2) accessory equipment employment of which further simplifies this procedure.

### CONSTRUCTION OF EQUIPMENT

A commercially available comparator block,\* shown in Fig. 1 was adapted for use in estimating the Ehrlich reaction by inserting sealed vials of pontacyl dye solution in the designated places and sealed vials of distilled water in the alternate positions. The pontacyl dye standards were prepared according to the directions of Watson and co-workers<sup>4</sup> in such a way that their color densities corresponded to those developed in the Ehrlich reaction by the designated concentrations of urobilinogen.<sup>5</sup> Flat bottomed tubes in the comparator block have the same diameter as the vials containing water and pontacyl dye. Their capacity is 7 ml. and they have a single mark at 5 milliliters. Tubes and vials of larger diameter would provide greater accuracy in the lower range of density but those here employed have the advantages of compactness and current availability.

Several types of simplified pipetting equipment were subjected to intensive use by medical students in the hospital laboratory and as a result of such trials the pipettes with attached rubber bulbs shown in Fig. 1 were deemed best suited for this purpose. The pipette used for measuring urine or fecal filtrate and that used for transferring Ehrlich's reagent from bottle to tubes have single marks at 1.25 milliliters. The pipette used in measuring and transferring the solution of sodium acetate has a single mark at 2.5 milliliters.

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\*The comparator block was obtained from the W. A. Taylor Company, Baltimore Md. through the Will Corporation, Rochester, N. Y. As a result of the observations described in this report, the Will Corporation is now in a position to supply comparator blocks already filled with vials of dye standards and water. The accessory pipettes, rubber bulbs, cork stoppers, and widemouthed bottles are also procurable from the Will Corporation. (The catalogue number for the entire unit is 17185.) The solutions of dye are stable and if contamination and unnecessary exposure to light are avoided the color densities should remain unchanged indefinitely.

## METHODS

The equipment described is used as follows in determinations of urobilinogen by the methods of Watson and associates<sup>4 5</sup>

*Two-Hour Specimens of Urine*—A two-hour urine specimen is collected in the middle of the afternoon when urinary urobilinogen excretion is usually maximal. The patient voids at 2 00 P M, discards the urine, drinks a glass of water, and voids at 4 00 P M. The volume of the latter specimen is recorded and the Ehrlich reaction is measured within one-half hour after voiding.

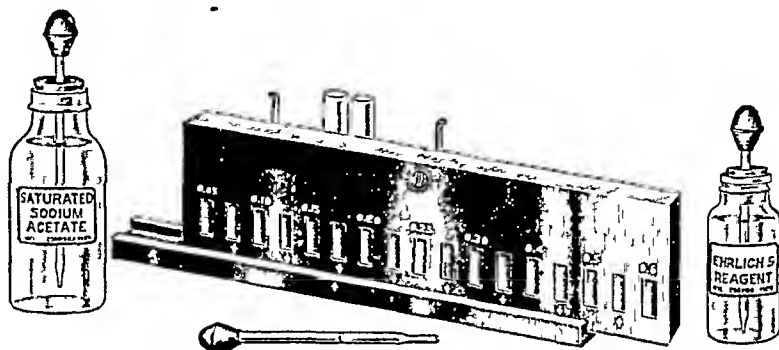


Fig 1—Comparator block and accessory equipment for measurement of urobilinogen

With the dropper pipette marked at 1.25 ml, this amount of urine is transferred to each of the two 5 ml tubes accompanying the comparator block. To one tube 1.25 ml of Ehrlich's reagent are added from the dropper bottle by use of the marked pipette. The contents are mixed by inversion and 2.5 ml of a saturated aqueous solution of sodium acetate are added without delay<sup>6</sup> and thoroughly mixed. To the other tube, which is to serve as the blank, the reagents are added to the urine in reverse order to prevent color development. First 2.5 ml of the solution of sodium acetate are added and the contents are mixed thoroughly, 1.25 ml of Ehrlich's reagent are then added slowly with constant shaking.

The blank and reaction tubes are placed in two adjacent openings in the comparator block and the sliding scale of dye standards is adjusted as necessary to bring the standard first selected for matching behind the blank and a vial of water behind the reaction tube as illustrated in Fig 2. If the match of colors obtained by holding up the set in front of a light is not satisfactory, a stronger or weaker color standard is moved into position behind the blank until the best match is found. Interpolation may be made if it is evident that the color intensity lies between two of the standards. If the blank develops a pink color or if the intensity of the unknown is higher than that of the color standard corresponding to a urobilinogen concentration of 0.6 mg per 100 ml further dilutions of the urine should be made, that is 1:2, 1:4, or 1:8, and so on, using the 1.25 ml drawn-out pipettes to measure urine and

distilled water into any ordinary test tube. The total amount of urobilinogen for the two hour period is calculated as follows:

Concentration of final solution in milligrams per 100 ml as determined from match with standards  $\times \frac{5}{125} \times \frac{\text{Milliliters urine excreted in 2 hours}}{100} \times \text{Any further dilution} = \text{Ehrlich units per 2 hours}$

Example  $0.20 \times 4 \times \frac{75}{100} = 0.60$  Ehrlich units per 2 hours

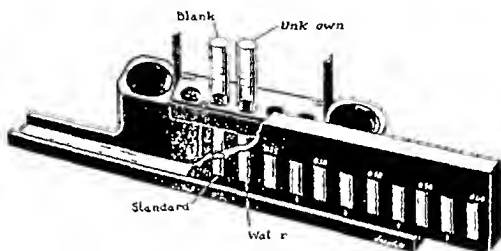


Fig 2—Cut away drawing of comparator block showing position of blank and unknown tubes in relation to tubes containing water and pontacyl dye standard

Normal adults usually excrete less than 10 Ehrlich unit during the two hour afternoon period. Results are expressed in Ehrlich units rather than in milligrams because nonurobilinogen chromogenic substances may contribute to the color measured by this simplified method.

If bilirubin is present in the urine it may interfere with the determination of urobilinogen, but in such cases the Ehrlich reaction can usually be carried out satisfactorily after merely diluting the urine with one or two parts of water. Porphobilinogen which is present in the urine of patients suffering from acute recurrent porphyria produces a pink aldehyde compound with Ehrlich's reagent, like that produced by urobilinogen. These two substances can be differentiated readily, however, by adding a few milliliters of chloroform to the reaction tube. The aldehyde compound of porphobilinogen remains in the aqueous fraction while that of urobilinogen is extracted by the chloroform, as shown by Watson and Schwartz.<sup>7</sup>

**Feces**—A 10 Gm aliquot of a single well mixed specimen is thoroughly ground with 90 ml of water. This is most easily accomplished by use of a Waring Blendor,<sup>8</sup> but an ordinary mortar and pestle will suffice. The fecal suspension is added to 100 ml of freshly prepared 20 per cent solution of ferrous sulfate (20 grams  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 92 \text{ cc H}_2\text{O}$ ) in a 500 ml Erlenmeyer flask. Unless the specimen is acholic, 100 ml of water are added after first being used to rinse out the vessel such as the Waring Blendor used in pre-

paring the suspension. One hundred milliliters of 10 per cent NaOH are then added slowly and with gentle shaking to the mixture in the Erlenmeyer flask. The flask is stoppered and placed in the dark for one hour, or until the supernatant solution is nearly colorless, at the end of which time a small portion of the mixture is filtered. The Ehrlich reaction is carried out on the filtrate in the same manner as with urine. Dilution of the filtrate with water (most conveniently 1:10) is usually necessary unless the concentration of urobilinogen in the stool is low. Calculation is carried out as follows:

Concentration of final solution as determined from match with standards  $\times \frac{5}{1.25} \times \frac{400 \text{ (or 300)}}{10} \times \text{Further dilution} = \text{Ehrlich units per 100 Gm. feces}$

Example  $45 \times 4 \times \frac{400}{10} \times 10 = 720$  Ehrlich units per 100 Gm. feces

Normal adults excrete from 50 to 300 Ehrlich units per 100 Gm. of feces. For more accurate measurements of hemoglobin metabolism or of the degree of biliary obstruction, it is advisable to collect stools over periods of from two to four days and to determine fecal urobilinogen excretion per diem by use of the method of Schwartz, Sborov, and Watson<sup>9</sup> which includes extraction with petroleum ether. The more elaborate method is also recommended for determinations of urobilinogen in twenty-four hour specimens of urine.

Watson and co-workers<sup>4</sup> found that measurements of urobilinogen by use of the comparator block do not differ significantly from those made with a photoelectric colorimeter employing a 565 m $\mu$  filter. Our experience with the commercially available equipment has been equally satisfactory. Biologic variations, difficulties in collection of specimens, and the presence of chromogenic substances other than urobilinogen<sup>10</sup> undoubtedly affect the results of these determinations to a considerable extent. Errors inherent in the use of the comparator block for routine clinical purposes are therefore considered inconsequential. Since matching of densities of color is most difficult in the lower range (0.1 mg. per 100 ml. and below), it is advisable to use dilutions of filtrate if possible which will produce densities comparable to those found in the higher range on the block.

In the Strong Memorial and Rochester Municipal Hospitals a comparator block and accessory equipment necessary for handling two hour urine specimens are placed in each divisional laboratory. The more elaborate equipment, including corked tubes containing 20 Gm. amounts of ferrous sulfate for preparing fecal filtrates, is located in a central laboratory. This equipment, which is used by interns and medical students, is kept in sturdy boxes in order to minimize breakage and exposure to light and dust. The boxes are inspected frequently, missing items replaced, and smeared comparator standards are cleaned with a damp cloth.

<sup>10</sup>Nonurobilinogen chromogenic substances are eliminated by the more elaborate method of Schwartz, Sborov, and Watson<sup>9</sup> in which stercobilinogen and mesobilirubinogen are extracted by petroleum ether from filtrates of urine or feces. In the present paper stercobilinogen and mesobilirubinogen are considered collectively as urobilinogen.<sup>10</sup>

The equipment described has proved most useful in making serial measurements on two hour specimens of urine. Such determinations usually can be made in about two or three minutes, even by inexperienced personnel. Although the results are not strictly quantitative, they are much more easily interpreted than those obtained by the serial dilution method of Wallace and Diamond.<sup>11</sup>

## SUMMARY

A comparator block and accessory equipment for measurement of urobilinogen in urine and stool are described. This equipment has the advantages of commercial availability and simplicity.

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# A SIMPLIFIED VACUUM DEHYDRATION TECHNIQUE FOR THE PREPARATION OF SECTIONS BY FREEZING-DRYING

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THE technique of drying frozen tissue in vacuo at reduced temperature for the preparation of microscopic sections requires apparatus which is too elaborate and expensive to be employed in many laboratories (Geish<sup>1</sup>) The procedure described below for the preparation of the frozen-dried sections involves a simplified method for dehydration which can be applied at a considerable economy of time and materials

## I APPARATUS (FIG 1)

1 A Cenco-Supervac pump of the mercury vapor diffusion type (*A*) This pump operates by the application of heat from an electric heater (*B*) to the mercury in the boiler (Method of use is described in the directions in Catalogue No 93201 for the steel Supervac pump of the Central Scientific Company)

2 Forepump (*C*) The diffusion pump requires a forepump capable of keeping the pressure in the condensing chamber sufficiently low to insure vaporization of the mercury The Cenco Hvyvac Pump, Catalogue No 91105, is recommended for this purpose These pumps are kept continuously functioning during the period of dehydration

3 The Supervac pump and forepump are connected by a glass tube (*K*)

4 Heater (*B*) The boiler of the pump should rest evenly in the circular opening of the heater (*B*) The heater should be put into operation after the mercury pump has been evacuated by the forepump and after the cold water has been started circulating through the water jacket

5 The dehydration chamber (*D*) and the surrounding Dewar flask (*F*) A Pyrex test tube (*D*) (diameter,  $2\frac{1}{2}$  cm, length, 20 cm) was used as the dehydrating chamber Drierite (8 mesh) ( $\text{CaSO}_4$ ) was placed in the bottom of the dehydrating tube to a height of about 2 cm as a drying agent to pick up the water as it comes off in the vapor state (*O*)

6 The connecting tube (*E*) A cylinder made of copper gauze containing a small amount of Drierite (*P*) was placed into the connecting tube (*E*) just before this tube was connected to the dehydrating tube *D*

7 The freezing trap (*G*) and the surrounding Dewar flask (*H*) In order to prevent mercury vapor from backing into the system being exhausted, a freezing trap (*G*) between the Supervac and the connecting tube (*E*) has been used This trap was surrounded by a mixture of solid carbon dioxide (dry ice) and acetone within a Pyrex silvered widemouthed Dewar flask (*H*), thus main

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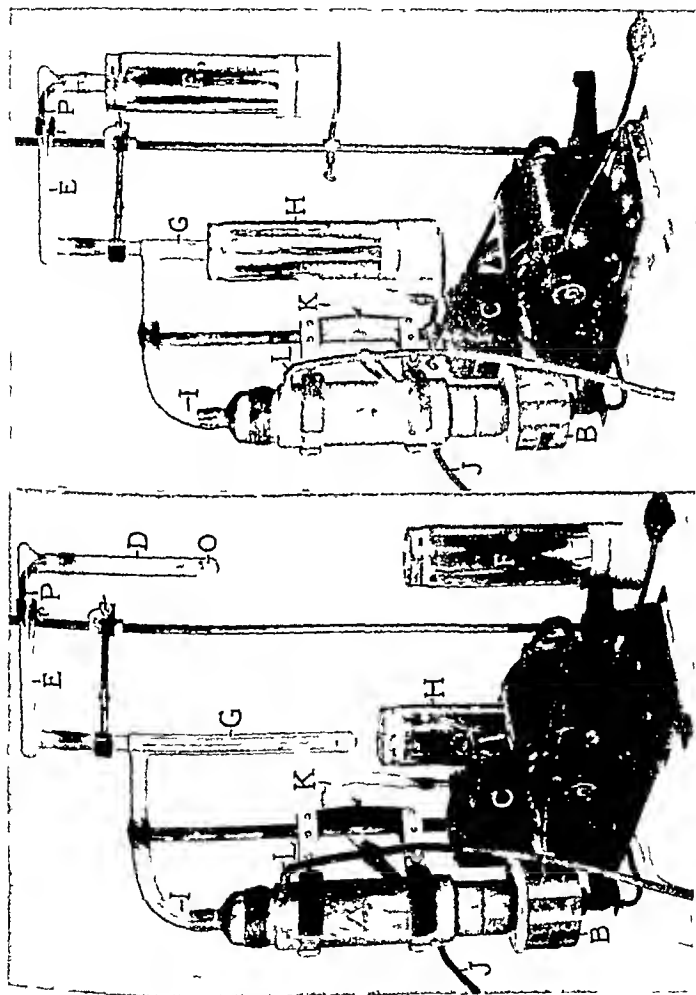


Fig. 1.—A Simplified apparatus for drying tissues from the frozen state. A Pyrex test tube. B Mercury diffusion pump. C Connecting tube connects dehydration chamber with freezing trap. D Freezing trap. E Dewar flask to surround freezing trap. F Vacuum pump. G Water outlet for condenser jacket of mercury pump. H Condenser jacket of mercury pump. I Water outlet for condenser jacket of mercury pump. J Copper gauze. K Drierite in copper gauze. L Spark coil after evacuating the system. All connections are made with para rubber tape and are tested for leaks with a spark coil after evacuating the system.

taining a temperature of about  $-80^{\circ}\text{C}$ . One end of the freezing trap was connected to the connecting tube (*E*), and the other end connected to the Supervac pump (*A*) by the connecting tube *I*.

8 All the connections should be made with the greatest care. After wrapping with para rubber tape (Cenco Catalogue No. 94220) the joint is covered with pure white shellac.

9 A high-frequency spark coil (Cenco Catalogue No. 80730) for testing vacuum assemblies for leakage was used after all the junctions were connected and the pumps had been started. Any leaks that were detected were repaired with additional rubber tape.

## II METHOD

1 Freezing. Tissue removed from the recently sacrificed animal was frozen in isopentane which was chilled in liquid air at  $-195^{\circ}\text{C}$  checked by a toluol thermometer. The isopentane should be stirred constantly to prevent it from becoming too viscous (Hoerr<sup>2</sup>). For most tissues dry ice and acetone ( $-80^{\circ}\text{C}$ ) can be substituted for the liquid air in cooling the isopentane.

2 Dehydrating. The frozen tissues were at once transferred to the drying tube (*D*), which had been cooled previously to  $-80^{\circ}\text{C}$  by solid carbon dioxide in the Dewar flask (*F*). During the transfer of the block to the cold dehydrating tube, the temperature of the block should not rise above  $-50^{\circ}\text{C}$ . After all the blocks are transferred to the dehydrating tube, it is then connected to the connecting tube (*E*) by para rubber tape, the forepump (*E*) is started and thirty minutes afterward the heater (*B*) of the mercury vapor pump (*A*) is turned on first starting cold water circulating through it at *J*.

3 To maintain the temperature of the vacuum dehydrating tube (*D*) at a temperature below  $-21^{\circ}\text{C}$ , we have used a very simple method. The method consists of surrounding the dehydrating tube with a Dewar flask (diameter,  $7\frac{1}{2}\text{ cm}$ , length, 26 cm) containing a large amount of solid carbon dioxide and a sufficient amount (near 2 lb) of dimethoxytetraglycol (Union Carbide and Carbon Corporation). Soon after mixing, this mixture will have a temperature of  $-80$  to  $-60^{\circ}\text{C}$ . The temperature of this mixture goes up gradually and becomes stabilized at  $-31^{\circ}\text{C}$  in from eight to twenty-four hours. Once the mixture reaches  $-31^{\circ}\text{C}$  it will maintain this temperature for a period of from two to three days. This property of dimethoxytetraglycol and dry ice mixtures was first pointed out by Wikoff<sup>3</sup>. The temperature should be checked with a toluol thermometer each day and a small amount of dry ice should be added when the temperature rises above  $-31^{\circ}\text{C}$ . An excess of dry ice should be avoided because it will bring the temperature below  $-31^{\circ}\text{C}$  and will prolong dehydration. Similarly a rise in temperature above  $-21^{\circ}\text{C}$  before dehydration is complete should be avoided, because collapse of the tissue and unsatisfactory fixation will result. In practice we have found that a single addition of a sufficient amount of dry ice to reduce the temperature to  $-31^{\circ}\text{C}$  on the third day of dehydration will result in good fixation for most specimens. The total length of the dehydration period is usually five days.

After the addition of dry ice on the third day, the temperature of the bath surrounding the dehydrating chamber is allowed to rise gradually toward room temperature usually reaching about  $-15^{\circ}\text{C}$  on the fifth day.

4 Most of the water removed from the tissue during dehydration will be condensed in the freezing trap (*G*) and a part of the water in the dehydration tube is absorbed by the Drierite in the drying tube (*O*). Only a small portion of the water vapor is absorbed by the Drierite (*P*) in the connecting tube (*E*).

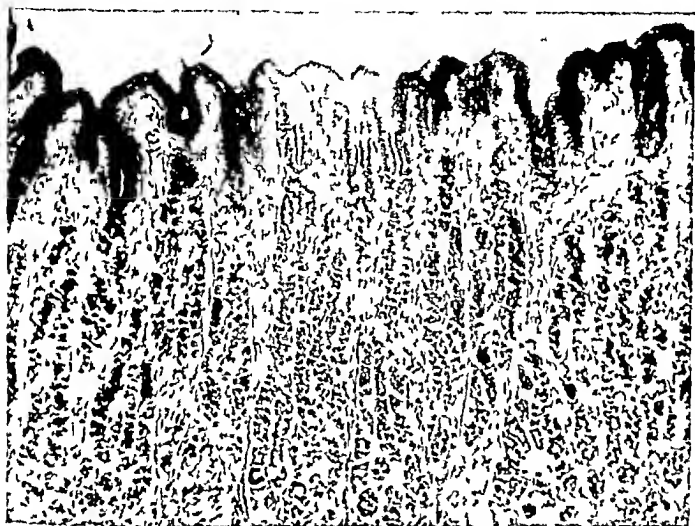


Fig. 2—Low power photograph of the gastric mucosa of dog frozen in isopentane at  $-195^{\circ}\text{C}$  dried in our vacuum system at  $-31^{\circ}\text{C}$ . Eight micron section stained by Hotchkiss method (polysaccharide staining with periodic acid). Shows uniformity of fixation and dehydration.  $\times 100$ .

5 When the drying is completed the Dewar flask (*F*) surrounding the dehydration chamber is removed and the electric heater on the mercury pump is turned off. The forepump is stopped about thirty minutes later. The dehydration tube (*D*) should be allowed to warm up to room temperature before the tissue is removed. Usually half an hour will be sufficient, so the dehydration chamber can be opened at the time the forepump is turned off.

6 Melted paraffin ( $58^{\circ}\text{C}$ ) is added to a warmed Erlenmeyer flask and the dehydrated tissues are placed in it to be infiltrated with paraffin by evacuation of the air in the flask by an ordinary oil pump, similar to the procedure of Gersh<sup>1</sup>. Embedding and cutting are done in the usual way. Fig. 2 is a photomicrograph of a section of dog's stomach prepared by this method.

## SUMMARY

A simplified method for the preparation of tissue sections by the method of freezing-drying is described. The method utilizes the constant temperature ( $-31^{\circ}\text{C}$ ) which a mixture of dry-ice and dimethoxytetraglycol will maintain over a long period of time as a means of holding the temperature of the tissue at a suitable level during dehydration.

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# STUDIES WITH COLLOIDS CONTAINING RADIOISOTOPES OF YTTRIUM, ZIRCONIUM, COLUMBIUM AND LANTHANUM

## I THE CHEMICAL PRINCIPLES AND METHODS INVOLVED IN PREPARATION OF COLLOIDS OF YTTRIUM, ZIRCONIUM, COLUMBIUM AND LANTHANUM

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### INTRODUCTION

FOR a number of investigations including fundamental studies of radiation effects on living tissues and therapeutic utilization of radioisotopes it is valuable to have methods for the selective localization of radioisotopes in certain tissues. Finely dispersed anhydrous chromic phosphate has been found useful by Jones, Wiobel and Lyons<sup>1</sup> in selectively irradiating the liver and spleen with  $P^3$  beta particles. The present studies, reported in this and the following communication,\* are concerned with methods for controlled selective localization of colloids (incorporating radioisotopes) in the liver, spleen, or bone marrow, and with an analysis of some of the factors involved in the phenomenon of localization.

*Choice of Elements for the Preparation of Colloids*—The elements zirconium, columbium, yttrium and lanthanum are particularly suitable for the present investigations. First it is possible to synthesize a variety of types of colloids incorporating radioisotopes of these elements. Second, there is available for these elements a large number of radioisotopes of differing nuclear properties. This group includes isotopes of short, intermediate, and long half-life, some of which emit only beta particles, some only gamma rays, and others both beta and gamma rays. Table I gives a description of the nuclear properties of the available radioisotopes.

*The Chemistry of the Preparation of Colloids of Zirconium, Yttrium, and Lanthanum*—Several types of colloids of the elements zirconium, yttrium, and lanthanum, varying in sign of charge, stability toward flocculation by added electrolytes, chemical structure and particle size may be prepared. As is shown in the following communication, both the disappearance rate of such colloids from the blood stream of animals following intravenous injection and the site of uptake of the colloids can be made to vary through wide limits by

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TABLE I RADIOISOTOPES OF YTTRIUM, ZIRCONIUM, COLUMBIUM, AND LANTHANUMS

ELEMENT	$T_{1/2}$	TYPE OF DECAY	ENERGY OF RADIATIONS IN MEV	
			BETA	GAMMA
Yttrium				
Y <sup>90</sup>	65 hr	$\beta$ , no $\gamma$	2.2	None
Y <sup>91</sup>	57 days	$\beta$ , no $\gamma$	1.53	None
Y <sup>93</sup>	105 days	K capture, $\gamma$	None	0.95 1.92
Zirconium				
Zr <sup>95</sup>	65 days	$\beta$ , $\gamma$	0.394 (98%)	0.73
Zr <sup>97</sup>	17 hr	$\beta$ , $\gamma$	1.0 (2%)	(0.92)?
Zr <sup>99</sup>	78 hr	$\beta^+$ , (?) $\gamma$	2.1 1.0	0.8 ?
Columbium				
Cb <sup>95*</sup>	90 hr	IT, e, $\gamma$ rays	e 0.22	(?)
Cb <sup>95</sup>	35 days	$\beta$ , $\gamma$	e 0.23 0.15	(Highly converted) 0.75
Lanthanum				
La <sup>140</sup>	40.0 hr	$\beta$ , $\gamma$	0.90 (20%) 1.4 (70%) 2.12 (10%)	0.335 (1%) 0.49 (7%) 0.83 (14%) 1.63 (74%) 2.3 (4%)

Note: None of the isotopes of half life less than 17 hours have been listed. All those described in this table are available from Oak Ridge or from cyclotron bombardment.

Symbols:  $\beta^-$  Electron emission  $\beta^+$  positron emission K K-electron capture IT isomeric transition e- conversion electron emission

alterations in the nature of the colloidal aggregates. The chemical principles involved and the methods used in the preparation of several of the colloids used in this work are given below.

#### A. Preparation of Zirconium Colloids of "Relatively Large" Particle Size

1. *Zirconium oxide peptized in dilute nitric acid.* Owens<sup>2</sup> has shown that freshly precipitated hydrated zirconium oxide may be resuspended in dilute nitric acid (insufficient stoichiometrically to dissolve the hydrated oxide) to produce positively charged colloidal zirconium oxide. The effective peptizing agent is probably the zirconyl ion produced by the reaction of the zirconium oxide with hydrogen ions.

*Preparation.* 0.5 ml of 0.1M zirconium oxychloride solution (containing Zr<sup>95</sup> or Zr<sup>99</sup> tracer) is titrated to the phenol red end point with dilute sodium hydroxide, resulting in the quantitative precipitation of the zirconium. The hydrated oxide precipitate is centrifuged out, the supernate being discarded. The precipitate is suspended in 2.5 ml of 0.02M nitric acid and then shaken twelve hours in a mechanical shaker to peptize the zirconium oxide. Following this the mixture is centrifuged three minutes at ~500 G. The supernate from this centrifugation is the final colloidal solution. This sol is visibly cloudy, demonstrates a bright Tyndall effect, is polydisperse, and of such particle size that the majority of the suspended zirconium is sedimented in ordinary centrifuges at ~2,000 G in sixty minutes. Before use in biologic experiments this sol is mixed with an equal volume of 10 per cent dextrose solution to render it approximately isotonic.

## 2 Zirconium phosphate in dilute disodium acid phosphate

*Preparation* 0.1 ml of 0.1M zirconium oxychloride is mixed with 2.3 ml of 5 per cent dextrose solution and to this mixture is added 0.1 ml of 0.6M disodium acid phosphite. There results a turbid sol which demonstrates a marked Tyndall effect on illumination. No evident flocculation of the colloid occurs even after thirty minutes of heating at 100° C.

Glucose is present for the purpose of rendering the sol nearly isotonic, since 0.9 per cent saline solution produces immediate flocculation of the colloidal zirconium phosphate. Should foreign ions flocculate the colloid, repeated extraction of the precipitate with the original volume of 5 per cent glucose solution containing disodium acid phosphite (0.024M) results in colloidal resuspension of the zirconium phosphate.

*3 Zirconium oxide peptized in sodium lactate solution* 0.5 ml of zirconium oxychloride solution is titrated to the phenol red end point with dilute sodium hydroxide, the hydrated zirconium oxide centrifuged out, and the supernate discarded. The precipitate is suspended in 2.5 ml of 0.008M sodium lactate solution and shaken twelve hours to peptize the hydroxide. Centrifugation for three minutes at ~500 G removes the largest aggregates, leaving a supernatant sol that is visibly cloudy and shows a marked Tyndall effect. The mechanism probably operative in production of this type of colloid, namely zirconium lactate complex ion formation is fully discussed in the next section. This colloid is mixed with an equal volume of 10 per cent dextrose solution before being used in biologic experiments.

*B Anionic Colloids of Zirconium and Yttrium of Varying Particle Size* Zirconyl ions form complexes in aqueous solutions with tartrate, lactate, and citrate ions of such stability thermodynamically as to prevent precipitation of the insoluble hydrated zirconium oxide even at relatively high hydroxide ion concentrations. Yttrium ions form complexes having similar properties with citrate ions.

It has been shown by Owens and Thomas<sup>3</sup> that the addition of such salts as sodium citrate, tartrate, or lactate to zirconium oxide hydrosols produces a marked rise in the pH of the hydrosol. This effect is attributed by them to the penetration of the micelles by the organic anions with replacement of hydroxide ions from the micelle, resulting in an increase in the free hydroxide ion concentration in the solution. Owens<sup>2</sup> has interpreted the conversion of positively charged hydrosols of zirconium oxide to negative sols in the presence of added citrate, tartrate, and lactate ions in the light of the above described complex ion formation and has shown further that by increasing the concentration of the complexing ion the colloidal aggregates may be broken down to particles diffusible through collodion membranes.

In our study of the zirconium hydroxide lactate (or tartrate citrate) and of the yttrium hydroxide citrate systems it has been found that at a particular pH (e.g., 7.0), by controlled variations of the mole ratio of complexing ion to metal ion added to the solution, aggregates are obtained from the extreme of rapidly sedimenting macroparticles through smaller and smaller



TABLE II VARIATIONS IN CHEMICAL COMPOSITION AND PARTICLE SIZE IN THE YTTRIUM HYDROXIDE CITRATE SYSTEM WITH CHANGES IN THE MOLE RATIO OF YTTRIUM TO CITRATE AT  $\text{pH} = 7$   
 [Total Volume = 10 ml Final  $\text{pH} \approx 7.0$  (Phenol Red End Point)]

	YTTRIUM* (MILLI MOLES) TOTAL PRESENT	CITRATE† (MILLI MOLES) TOTAL PRESENT	HYDROXIDE (MILLI MOLES) BOUND	HYDROXIDE (MILLI MOLES) BOUND PER MILLIMOLE YTTRIUM	APPEARANCE OF SOLUTION IN THE FIRST THREE HOURS AFTER TITRATION
1	0.2	0.0	0.03	0.15	
2	0.2	0.025	0.15	0.70	Rapidly sedimenting precipitate with clear supernate
3	0.2	0.05	0.22	1.1	Rapidly sedimenting precipitate with clear supernate
4	0.2	0.07	0.22	1.1	Rapidly sedimenting precipitate with clear supernate
5	0.2	0.08	0.22	1.1	Rapidly sedimenting precipitate with clear supernate
6	0.2	0.09	0.23	1.2	Rapidly sedimenting precipitate with clear supernate
7	0.2	0.10	0.28	1.4	Slowly sedimenting precipitate with supernate showing moderate Tyndall effect
8	0.2	0.015	0.19	0.95	Very slowly sedimenting precipitate with supernate showing marked Tyndall effect
9	0.2	0.125	0.18	0.90	
10	0.2	0.138	0.19	0.90	Intense Tyndall effect in supernate with minimal quantity of very slowly settling precipitate
11	0.2	0.15	0.19	0.90	Moderate Tyndall effect, no precipitate
12	0.2	0.20	0.15	0.75	Faint Tyndall effect, no precipitate
13	0.2	0.25	0.10	0.50	Clear solution, no precipitate
14	0.2	0.30	0.062	0.31	Clear solution, no precipitate
15	0.2	0.35	0.035	0.18	Clear solution, no precipitate
16	0.2	0.40	0.013	0.065	Clear solution, no precipitate
17	0.2	0.45	0.0084	0.042	Clear solution, no precipitate
18	0.2	0.50	0.0056	0.028	Clear solution, no precipitate
19	0.2	0.55	0.0016	0.0080	Clear solution, no precipitate
20	0.2	0.60	0.00	0.000	Clear solution, no precipitate

\* Added as a solution of yttrium nitrate in water

† Added as sodium citrate solution

From the data in Table II it is seen that on reaching the ratio citrate/yttrium = 3 there is no hydroxide bound by yttrium. As would be expected from equilibrium considerations this continues to be the case at even higher citrate/yttrium ratios

particles to aggregates whose sols (or solutions) no longer give a Tyndall effect. In any one of these preparations, the number of moles of hydroxide ion bound per mole of metal (zirconium or yttrium) varies with the number of moles of complexing ion present. Table II gives the titration values (moles hydroxide bound per mole yttrium) for yttrium in the presence of various total quantities of citrate ions (bound plus free) at  $\text{pH} 7$ . At the mole ratio yttrium/citrate = 2, and at  $\text{pH} 7$ , where 1.4 moles of hydroxide are bound per mole of yttrium one obtains rapidly sedimenting macroparticles. As the citrate/yttrium mole ratio is raised, the number of moles of hydroxide bound per mole of yttrium decreases sharply, and simultaneously the aggregate size drops from particles sedimenting rapidly, through those sedimenting more and more slowly then through those whose sols show an intense Tyndall effect without sedimentation at fields of  $\sim 2,000$  G down to those whose sols (or solutions) produce no Tyndall effect. Similar data for the yttrium-hydroxide citrate system at a final  $\text{pH}$  of  $\sim 8.3$  (phenolphthalein end point) are given in Table II.

TABLE III VARIATION IN CHEMICAL COMPOSITION AND PARTICLE SIZE IN THE YTTRIUM HYDROXIDE CITRATE SYSTEM WITH CHANGES IN THE MOLE RATIO OF YTTRIUM TO CITRATE AT pH = 8.3  
[Total Volume = 10 ml Final pH  $\approx$  8.3 (Phenolphthalein End Point)]

	YTTRIUM (MILLI MOLES) TOTAL PRESENT	CITRATE (MILLI MOLES) TOTAL PRESENT	HYDROXIDE (MILLI MOLES) BOUND	HYDROXIDE (MILLI MOLES) BOUND PER MILLIMOLE YTTRIUM	APPEARANCE OF SOLUTION IN THE FIRST THREE HOURS AFTER TITRATION
1	0.2	0.0	0.356	1.88	
2	0.2	0.025	0.39	1.96	Rapidly sedimenting precipitate, with faint Tyndall effect in supernate
3	0.2	0.05	0.34	1.72	Rapidly sedimenting precipitate plus strong Tyndall effect in supernate
4	0.2	0.075	0.300	1.50	No precipitate liquid shows strong Tyndall effect
5	0.2	0.10	0.28	1.39	No precipitate no Tyndall effect in liquid
6	0.2	0.125	0.268	1.34	No precipitate no Tyndall effect in liquid
7	0.2	0.15	0.247	1.25	No precipitate no Tyndall effect in liquid
8	0.2	0.175	0.246	1.23	No precipitate no Tyndall effect in liquid
9	0.2	0.20	0.233	1.19	No precipitate no Tyndall effect in liquid
10	0.2	0.25	0.229	1.14	No precipitate, no Tyndall effect in liquid
11	0.2	0.30	0.23	1.16	No precipitate no Tyndall effect in liquid
12	0.2	0.35	0.221	1.10	No precipitate no Tyndall effect in liquid
13	0.2	0.40	0.21	1.08	No precipitate no Tyndall effect in liquid
14	0.2	0.45	0.218	1.09	No precipitate no Tyndall effect in liquid
15	0.2	0.50	0.205	1.02	No precipitate no Tyndall effect in liquid
16	0.2	1.00	0.204	1.02	No precipitate, no Tyndall effect in liquid
17	0.2	4.00	0.188	0.94	No precipitate no Tyndall effect in liquid

On comparing data in Table II with that in Table III it is seen that the hydroxide binding to yttrium is much more stable at pH 8.3 than at pH 7.0, so that at the higher pH even with citrate/yttrium ratios of 20:1, there is still approximately 1 millimole hydroxide bound per millimole of yttrium. Further, on comparing the products obtained at any particular citrate/yttrium ratio by using the pH from 7.0 to 8.3, it is noted that in addition to changing the extent of hydroxide/yttrium binding the particle sizes are smaller at pH 8.3 than at pH 7.0 (at least through the range of mole ratio of citrate/yttrium from 1.4 to 1.1). Qualitative experiments reveal that on still further increasing the pH (i.e. above 8.3) a region is reached where the aggregate size begins again to increase with pH increase.

It is evident from the above mentioned data that it is possible to prepare colloidal sols whose particle sizes may be made to vary through a wide range by altering the citrate/yttrium mole ratio at a particular pH. Or if a particular citrate/yttrium ratio is chosen one can vary particle sizes by altering the final solution pH. The aggregates obtained by the second of these procedures

are chemically different from those obtained by the first procedure, as is evident from the increased hydroxide binding at pH 8.3 compared with that at pH 7.0. Colloids prepared on the basis of these data may be used to investigate both the effect of particle size and of chemical structure upon such physiologic properties as blood stream clearance, rapidity of phagocytosis by elements of the reticulo-endothelial system, and site of uptake of the colloids *in vivo*.

In the case of zirconyl ions, in a system with hydroxide and citrate, tartrate, or lactate, a set of data similar to those for yttrium may be obtained by varying the ratio of complexing ions to zirconyl ion at any particular pH. One factor which differs is that the reaction between zirconyl ions and hydroxide ions at room temperature and above produces some bonds that, once formed, are very slowly broken by back titration. As a result, care must be exercised in preparing colloids of zirconium to add alkali slowly with adequate stirring, in the cold, to avoid such an occurrence during the process of titrating to the desired final pH value.

For several investigations described in the subsequent communication, colloidal preparations of zirconium and yttrium prepared according to the above-described principles have been used. In the case of both these elements, the colloidal particles thus prepared are much smaller than those in the preparations described in the section on "relatively large particle size" colloids. These latter sols, prepared according to the following directions, show only a faint Tyndall effect, and the particles are nonsedimentable using the centrifugal fields obtainable with ordinary centrifuges (2,000 G). Studies are now in progress to obtain a quantitative measure of particle sizes in such preparations as well as information as to particle symmetry and the extent of polydispersity of the sols. For the present the designation of "relatively large" particles and "intermediate" particles will be used without commitment as to actual particle diameters.

*C Preparation of Zirconium-Hydroxy-Lactate Colloid of "Intermediate" Particle Size* One milliliter of 0.4M zirconium oxychloride solution (containing the desired zirconium radioisotope) is mixed with 1.2 ml of 1.0M sodium lactate solution, 1 drop phenol red indicator solution is added, and the mixture diluted to 6.0 ml with water. This solution is then titrated carefully with 1M NaOH to the phenol red end point, always maintaining the temperature near 0° C. On heating this solution at 100° C the indicator color reverts to yellow. After ten minutes at 100° C, the solution is cooled to 0° C and again brought to the phenol red end point with NaOH. The alternate heating and cooling with repeated titration is continued until the end point (color) persists even after ten minutes of heating. This final solution is then diluted to 10 ml, heated at 100° C for ten minutes, and cooled. During the course of the titration to the phenol red end point it is imperative to avoid additions of alkali while the solution is warm for reasons previously mentioned in the general discussion. Colloids prepared according to these directions maintain their original properties at least over a period of two months when stored in ordinary rubber-capped Pyrex vessels.

*D Preparation of Yttrium Hydroxy Citrate Colloids* 0.5 ml of 0.2M  $Y(NO_3)_3$  solution (incorporating the desired yttrium radioisotope) is mixed with 0.16 ml of 0.5M sodium citrate solution, 1 drop phenol red indicator is added, and the mixture diluted to 2.0 ml with water. This solution is heated five minutes at  $100^\circ C$ . A precipitate will form at this step, or before heating, but the procedure is carried on without separating the precipitate, which later resuspends spontaneously. The solution is then titrated with 1.0M NaOH to the phenol red end point. On reaching the end point the solution is heated at  $100^\circ C$  for five minutes, cooled in ice water bath and if the end point has faded, is re-titrated with sodium hydroxide. In contrast with the zirconium procedure, this re-titration is necessary only once. The final solution is diluted to 4.0 ml and then heated one hour at  $100^\circ C$  in a rubber capped Pyrex serum bottle. Such colloidal preparations show no change in appearance or biologic properties over a period of four to six months. These colloids have been heated as long as eight hours at  $100^\circ C$  without demonstrating any change in properties.

*E Colloids of Lanthanum* Similar procedures have been utilized to prepare colloids in the lanthanum hydroxide citrate system, the qualitative details in this system being similar to those of the yttrium hydroxide citrate system, but with differences existing in the thermodynamic stability of the bonds involved. Since we have not yet studied the biologic properties of lanthanum colloids, the detailed chemical preparation is not given.

*F Colloids Containing Columbium* In using the radioisotope  $Zr^{93}$  (65 day half life), there is the problem of the 35 day  $Cb^{93}$  daughter isotope to consider. A chemical separation of the two isotopes can be made, but of course the  $Cb^{93}$  will soon regrow in the  $Zr^{93}$  preparation. We have investigated the zirconium colloids described in this communication using the  $Zr^{93}$  isotope (with a stable yttrium daughter), and using equilibrium mixtures of  $Zr^{90}$  and  $Cb^{90}$ . No quantitative differences in biologic behavior have been found, so that for these particular studies it appears that if the  $Cb^{93}$  isotope (without carrier) is incorporated into the zirconium colloid it shares the same metabolic fate as does the  $Zr^{93}$  isotope. For this reason no separation of these isotopes need be made for the particular purposes described in the following communications.

#### SUMMARY

1 For studies concerned with selective localization of radioisotopes in the bone marrow, liver, and spleen colloids incorporating such isotopes are useful.

2 Colloids of zirconium, columbium, yttrium, and lanthanum have been chosen for study of selective localization of colloids because of the availability of radioisotopes of a wide range of nuclear properties and because of the comparative ease with which controlled variations in chemical structure and particle size of colloids containing these elements may be made.

3 The methods for preparing zirconium and yttrium colloids of various chemical types and ranges of particle size are described. A discussion of some of the chemical principles of these preparative methods is given.

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# STUDIES WITH COLLOIDS CONTAINING RADIOISOTOPES OF YTTRIUM, ZIRCONIUM, COLUMBIUM, AND LANTHANUM

## II THE CONTROLLED SELECTIVE LOCALIZATION OF RADIOISOTOPES OF YTTRIUM, ZIRCONIUM, AND COLUMBIUM IN THE BONE MARROW, LIVER, AND SPLEEN

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### INTRODUCTION

SALFRAAL workers have shown that certain colloiddally dispersed materials are removed from the blood stream by the liver and spleen. Jones, Wiobel, and Lyons<sup>1</sup> have utilized suspensions of anhydrous chromic phosphate for the selective irradiation of the liver and spleen with  $P^3$  beta particles. Gersh<sup>2</sup> demonstrated that colloidal calcium phosphate is taken up by the liver and spleen. He stressed the failure of bone marrow phagocytes to take up this colloid in rats and dogs (though he referred to possible uptake in the marrow of rabbits under special conditions) and commented on the relative "refractiveness" in general of the bone marrow as compared with liver and spleen with respect to the uptake of colloidal dyes from the blood stream. Some histologic data<sup>3</sup> indicate that Thorotrast (a colloidal thorium dioxide preparation) is deposited in the bone marrow as well as in the liver and spleen, but no quantitative data as to the relative distribution are available.

In the preceding communication\* the methods for the preparation of colloids incorporating radioisotopes of yttrium, columbium, and zirconium were given. The present studies are concerned with the localization of such colloids primarily in the bone marrow or primarily in the spleen and liver, with an analysis of some of the factors which may be responsible for differences in localization.

1. *Selective Localization of Radioisotopes of Zirconium, Columbium, and Yttrium in the Liver and Spleen*—Several different colloids have been studied, all of which show the common property of localizing in the liver and spleen of mice, rats, and rabbits. In every case the colloids having this distribution are those of "relatively large" particle size, sedimentable in large part in ordinary centrifuges. These sols are turbid and produce an intense Tyndall effect on illumination. Further, in general, for the colloids which localize in the liver and spleen, the disappearance rate of intravenously injected colloid from the blood stream is rapid, the blood colloid level decreasing approximately exponentially with time, the half time for blood clearance (and liver

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TABLE I DISTRIBUTION OF INTRAVENOUSLY INJECTED ZIRCONIUM COLLOIDS OF RELATIVELY LARGE PARTICLE SIZES IN MICE ( $Zr^{90}$  AND  $Ce^{95}$  RADIOISOTOPES INCORPORATED INTO COLLOID)

ORGAN	ZIRCONIUM OXIDE IN DILUTE $HNO_3$ (1) (% INJECTED DOSE)	ZIRCONIUM PHOS- PHATE IN DILUTE $NaHPO_4$ (2) (% INJECTED DOSE)	ZIRCONIUM OXIDE IN DILUTE SODIUM LACTATE (3) (% INJECTED DOSE)
Liver plus spleen*	97.3	90.8	95.0
Lungs	0.6	0.9	Included with carcass
Blood	0.4	1.8	0.7
Entire remainder of carcass including bones	1.7	6.5	4.5

(1) Mouse sacrificed six minutes after injection

(2) Mouse sacrificed three minutes after injection

(3) Mouse sacrificed five minutes after injection

\*Data on many animals for spleen alone show uptakes varying between 1 per cent and 4 per cent of injected dose

#### BLOOD STREAM DISAPPEARANCE FOR "RELATIVELY LARGE PARTICLE SIZE" COLLOID IN THE RABBIT

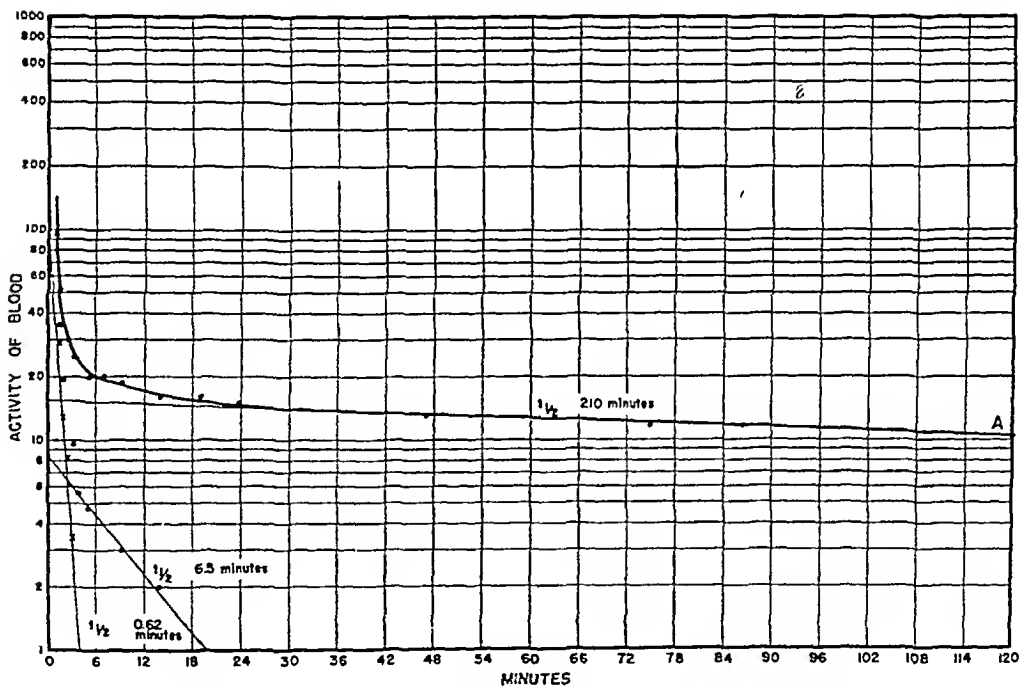
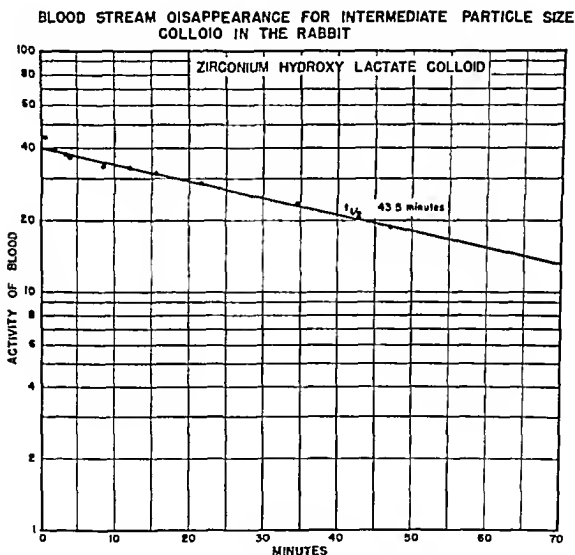


Fig 1

and spleen uptake) being in the neighborhood of 30 seconds to 1 minute. Representative distribution data for the three colloids—(1) zirconium oxide peptized in nitric acid, (2) zirconium phosphate peptized in disodium acid phosphate and (3) zirconium oxide in dilute sodium lactate—in the mouse are given in Table I. The preparative procedure for these colloids is given in the preceding communication.

In the case of all of these colloids the blood disappearance curves show a small percentage (between 1 and 10 per cent) of more slowly clearing components in addition to that component having the  $T_1$  of 30 seconds to 1 minute. The methods of preparation for these sols are such that polydispersity of the sols is expected. Several types of experimental data lend support to the idea that the smaller particles in such polydisperse sols are responsible for the slowly clearing fraction. First, experiments have been performed, using the mouse, where a colloid having  $\sim 1$  per cent of the total activity in the slowly disappearing fraction has been centrifuged to remove the larger particles



The supernatant colloid was then injected and was shown to have a slowly disappearing fraction of  $\sim 8$  per cent. These data appear to support the idea that the smaller particles constitute the slowly disappearing fraction. Further experiments with a colloid of zirconium oxide suspended in sodium lactate were done in the rabbit. Fig 1 gives the blood disappearance curve\*. This particular preparation has an appreciable percentage of slowly clearing components in addition to the major fraction of very rapid blood clearance rate.

The rapid disappearance rate cannot be interpreted as simply due to mixing phenomena, since much evidence accumulated shows that in the rabbit mixing phenomena are of negligible proportions after one minute. The data used in Fig 1 demonstrate rapid disappearance after this mixing period. Figs 2 and 3 demonstrate that mixing phenomena do not result in an apparently rapid disappearance curve at times after one minute following injection.



BLOOD STREAM DISAPPEARANCE FOR INTERMEDIATE PARTICLE SIZE COLLOID IN THE RABBIT

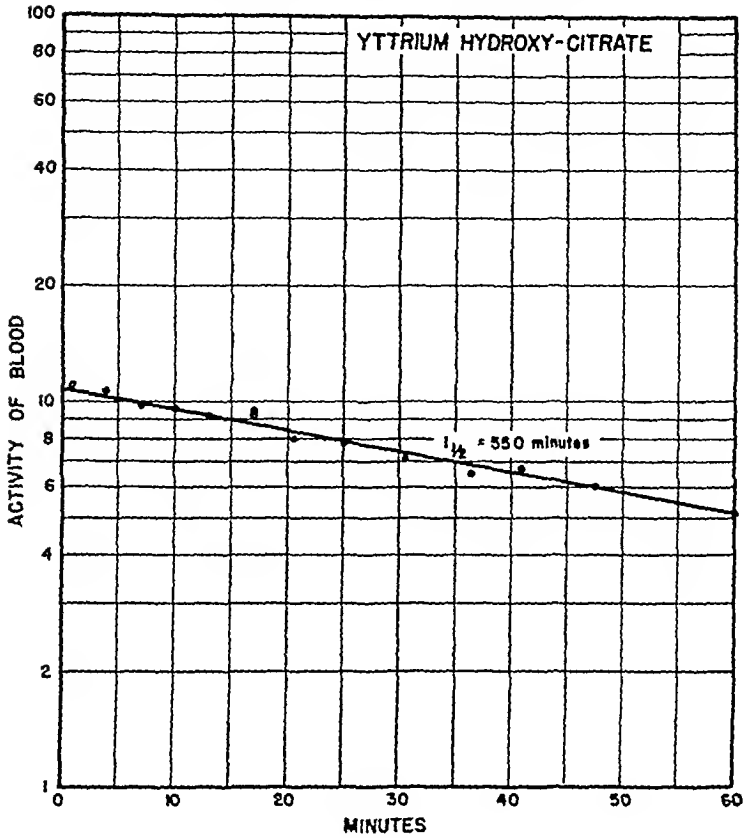


Fig 3

At two hours after injection (A, Fig 1), when all the rapidly disappearing fraction had been cleared, serum was collected and the rabbit sacrificed. The serum containing the slowly disappearing component was injected into a second rabbit. The second rabbit was sacrificed after its blood had been cleared, and the tissue distribution of the colloid was determined. The data are given in Table II.

TABLE II

ORGAN	DISTRIBUTION OF ZIRCONIUM COLLOID IN INITIAL RABBIT (INCLUDING RAPIDLY CLEARING FRACTION) (% INJECTED DOSE IN ENTIRE ORGAN)	DISTRIBUTION OF ZIRCONIUM ACTIVITY IN ANIMAL INJECTED WITH SERUM FROM INITIAL ANIMAL (% INJECTED DOSE IN ENTIRE ORGAN)
Bone plus marrow	93	43.0
Liver	85.0	23.0
Spleen	3.2	3.0
Muscle	1.7	17.0
Lungs	1.0	---
Kidney	0.3	14.4

It is seen that the liver received the major fraction of the rapidly clearing colloid, whereas the bone plus bone marrow received the major fraction of the slowly clearing colloid component. Thus in all the experiments tabulated (and in several others for similar colloids) where the colloidal particles are relatively large in size sedimentable in large part even with ordinary centrifuges producing sols that are turbid and demonstrate an intense Tyndall effect we have found the blood clearance rate to be very rapid ( $T_1 = 30$  seconds to 1 minute) and the site of uptake of the colloid to be the liver and spleen primarily. From the magnitude of the blood clearance rate it is certain that much of the colloid is cleared in a single passage through the liver so that for these colloids the rate of blood flow through the liver relative to that through the bone marrow probably plays a major role in securing localization of the colloid in the liver plus spleen rather than in the bone marrow. For chromic phosphate suspensions also it is now known that there is association of relatively large particle size rapid blood clearance and liver and spleen localization just as with the above described zirconium colloids. The mechanism responsible for the rapid uptake by the liver and spleen of relatively large particles is not yet clear nor has the significance of factors other than particle size alone been evaluated in determining blood clearance rate and site of uptake of the colloids.

*B Selective Localization of Radioisotopes of Zirconium, Columbium and Yttrium Primarily in the Bone Marrow, and Secondarily in the Spleen and Liver*—As described in the preceding communication zirconium colloids of zirconium and yttrium may be prepared of much smaller particle size than those just shown to deposit primarily in the liver plus spleen. These colloids are of the zirconium hydroxy lactate and the yttrium hydroxy citrate type. In contrast to the rapid blood clearance of the "relatively large size" aggregates these latter colloids of intermediate particle size are cleared much more slowly in the rabbit, rat and mouse showing exponential decay from the blood stream with half times from 30 to 80 minutes (see Figs. 2 and 3) the colloid remaining quantitatively in the plasma until cleared. In the rabbit, rat, and mouse the slowly disappearing colloids localize primarily in the bone marrow, with spleen and liver receiving the next highest quantity (all expressed on specific activity basis)\*. The distribution data in the rabbit for the zirconium hydroxy lactate colloid are given in Table IV and for yttrium hydroxy citrate colloid in Table III. In the case of zirconium and yttrium colloids which localize primarily in the bone marrow we find associated these features: (1) particle size much smaller than in those colloids localizing primarily in the spleen and liver (less pronounced Tyndall effect noncentrifugibility with ordinary centrifuges) (2) slow disappearance from the blood stream ( $T_1 = 30$  to 80 minutes contrasted with 30 seconds to 1 minute for "liver and spleen" colloids).

\* In the mouse the liver and spleen take up the major fraction of the slowly disappearing colloids although the bone marrow uptake is greater than for the large particle colloids.

TABLE III DISTRIBUTION OF INTRAVENOUSLY INJECTED YTTRIUM HYDROXY CITRATE COLLOID IN THE TISSUES OF THE RABBIT ("INTERMEDIATE" PARTICLE SIZE)  
(Half Time for Blood Stream Clearance = 70 min)  
(Animal Sacrificed 18 hr After Injection)  
(Y<sup>91</sup> Tracer — 1.0 cc Injection)

ORGAN	% OF INJECTED DOSE IN ENTIRE ORGAN	% OF INJECTED DOSE PER GRAM OF TISSUE (SPECIFIC ACTIVITY)
Bone and marrow	35.0	0.07
Bone (rib)*	---	0.21
Bone (femur)*	---	0.11
Marrow (rib)	---	0.45
Marrow (femur)	---	0.71
Spleen	1.0	0.67
Liver	45.0	0.22
Kidneys	1.2	0.056
Urine (first 18 hr after injection)	3.6	---
Gastrointestinal tract	2.4	0.013
Heart	0.12	0.057
Lymph nodes	---	0.01
Muscle	0.54	0.0003
Testes	0.05	0.006
Brain	0.02	0.002
Lungs	0.53	0.02

\*It is difficult mechanically to remove all the marrow so that bone specific activities always tend to be falsely high when most of the colloid is in the marrow

†The specific activity is the really significant value for calculation of irradiation dose received by a tissue following localization

As was shown in the preceding communication, it is possible to prepare particles of different chemical composition (but of the same size range) by altering the final solution pH at a particular metal ion/complexing ion ratio in the yttrium-hydroxide-citrate system. With quantitative measure of par-

TABLE IV DISTRIBUTION OF INTRAVENOUSLY INJECTED ZIRCONIUM\* HYDROXY LACTATE COLLOID IN THE TISSUES OF THE RABBIT  
(Half Time for Blood Stream Clearance = 70 min)  
(Animal Sacrificed 24 hr After Injection)

ORGAN	% OF INJECTED DOSE IN ENTIRE ORGAN	% OF INJECTED DOSE PER GRAM OF TISSUE (SPECIFIC ACTIVITY)
Bone and marrow	44.0	0.11
Bone (femur)	---	0.03
Marrow (femur)	---	0.84
Spleen	1.3	0.65
Liver	37.0	0.28
Kidneys	2.7	0.15
Heart	0.09	0.045
Lungs	0.04	0.022
Urine (first 4 hr after injection)	3.2	---
Muscle	1.2	0.0007
Lymph nodes (~ 60% of total)	0.5	---
Gastrointestinal tract	3.0	0.018
Thymus	0.025	---
Pituitary	0.001	---
Adrenals	0.002	---
Brain	0.01	0.0014
Eyes	0.1	0.014

\*A mixture of Zr<sup>90</sup> and Cb<sup>90</sup> radioisotopes was used in preparation of this colloid. The distribution data differed in no significant details from that for Zr<sup>90</sup>, a positron emitter, the yttrium daughter of which is stable, indicating that the Cb<sup>90</sup> radioisotope shares the metabolic fate of the zirconium colloid.

particle sizes of these types, it is planned to try to gain some insight into the possible effect of variations in chemical composition (at the same particle size) on blood stream disappearance rate and site of uptake of colloids, and thus perhaps obtain a clearer idea of the mechanism operative in selective localization in one element or another of the reticulo-endothelial system. From the present data it is not possible to determine whether or not polymerization of the slowly disappearing colloids is a necessary preliminary to reticulo-endothelial deposition or whether the particles remain in the serum at the original size and are slowly removed at this size.

*C Retention of Yttrium and Zirconium Colloids in Tissue Following Initial Uptake*—For several applications of such colloids in selective irradiation it is necessary to know to what extent the colloids remain fixed in tissues once deposited.

TABLE V PATENT OF RETENTION OF YTTRIUM HYDROXY CITRATE AND ZIRCONIUM HYDROXY LACTATE COLLOIDS IN TISSUES OF DEPOSITION IN THE RABBIT

ORGAN	INJECTED DOSE STILL PRESENT IN ENTIRE ORGAN					
	YTTRIUM HYDROXY CITRATE COLLOID		ZIRCONIUM HYDROXY LACTATE COLLOID			
	DAYS AFTER INJECTION					
	2/3	11	1/0	1	10	22
Bone and marrow	3.0	53.0	44.0	46.0	35.0	34.0
Spleen	1.0	0.4	1.3	2.9	1.8	1.1
Liver	45.0	29.0	37.0	21.0	24.0	22.0
Kidneys	1.2	0.7	2.7	2.0	3.1	2.3
Lungs	0.5	0.3	0.4	0.9	0.6	0.3

\*Data in this table were obtained by injecting separate rabbits with the colloids and sacrificing the animals at the stated time intervals after injection.

For the zirconium phosphate colloids (large particle type) in the mouse, analysis shows that there is at ten days after injection 96.5 per cent of injected zirconium in liver plus spleen, 0.3 per cent in the lungs, and 3.3 per cent in the entire remainder of the carcass. Comparison of these data with those for the same colloid in Table I shows that no significant loss of activity or change in distribution occurs for this colloid over this time interval.

Similar data for the yttrium hydroxy citrate and zirconium hydroxy lactate "intermediate particle size" colloids are given in Table V.

Excretion data in the rabbit show negligible losses of yttrium colloid via the urine after the first day while  $\frac{1}{2}$  to 1 per cent of the injected dose is lost daily via the feces.

Except for a possible trend toward slow loss of activity from the liver, it is seen that the bulk of the yttrium and zirconium colloids tend to remain localized at the site of deposition, at least for the periods of time given in Table V.

*D Experimental and Therapeutic Applications*—For experimental work requiring specific irradiation of the liver, spleen, or bone marrow or combinations of these organs the colloids described are useful. With the range of short and long lived radioisotopes of yttrium, zirconium, and columbium

available and with the evidence presented that colloids incorporating such isotopes may be retained in tissues of deposition for at least several weeks, it is possible to achieve continuous specific irradiation of tissue at any desired intensity level either for short or long periods of time

In certain neoplastic diseases of the hematopoietic system, as the leucemias, myelomas, or other neoplasms involving the liver or bone marrow, and in polycythemia, the therapeutic use of yttrium or zirconium colloids seems feasible. With these colloids irradiation is localized in these tissues while minimizing the irradiation of other tissues. With a range of half lives of beta-emitting isotopes from 170 hours to 65 days, one may have a great latitude in choosing duration and intensity of therapeutic irradiation. A clinical evaluation of these colloids, with incorporated radioisotopes, is now being made both with respect to tissue distribution in human beings and with respect to therapeutic efficacy in polycythemia, the leucemias, and certain other selected diseases. While no great differences from other forms of radiation therapy are anticipated, it is possible that some improvement in the management of these diseases may result.

#### SUMMARY

1 Colloids of zirconium of relatively large particle size show rapid disappearance from the blood stream ( $T_1 = \sim 30$  seconds to 1 minute) and are deposited mainly in the liver and spleen.

2 Colloids of smaller particle size, both of zirconium and yttrium, disappear much more slowly from the blood stream ( $T_1 = 30$  to 80 minutes) and are deposited primarily in the bone marrow and spleen, secondarily in the liver—the liver specific activity being approximately one-third that of marrow.

3 Both types of colloids, once deposited in these organs, show no significant change in distribution pattern, at least over a period of two to four weeks, and only slow excretion from the body.

4 The distribution of the colloids and the availability of the isotopes of a wide range of nuclear properties render them suitable for experimental studies requiring specific irradiation and for therapeutic utilization in certain diseases.

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# AN EXPERIMENTAL STUDY OF THE EFFECT OF ZIRCONIUM AND SODIUM CITRATE TREATMENT ON THE METABOLISM OF PLUTONIUM AND RADIOTRITIUM

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## INTRODUCTION

THE routine processing and handling of large and ever increasing quantities of radioelements are now in actuality. When these radioelements find their way into the body they may cause radiation injury in a manner similar to radium poisoning. Internally fixed radioelements are of special concern since the amounts needed to produce injury are trifling compared with the quantities necessary to cause equivalent damage from external sources of radiation. Most of these potentially dangerous radioelements are metals. The purpose of this and subsequent studies is the development of rapid, safe, and effective procedures for increasing the elimination of potentially harmful radioelements which have gained entry into the body. The magnitude and nature of the problems involved have been described ably by Hamilton and associates.<sup>1, 2</sup>

It has been postulated and shown that the excretion of certain metallic elements fixed in the body should be increased following the administration of relatively large amounts of the soluble salts of certain other metals. The citrate salt of zirconium when given to rats was found to cause a considerable increase in the urinary excretion of plutonium and a large decrease in the amount of plutonium deposited in the skeleton. An investigation of the effects of zirconium citrate and salts of other metals on the excretion and distribution of a large variety of radioelements is now being conducted in this laboratory. The present study provides a direct comparison of the very marked displacing action of zirconium citrate on plutonium ( $\text{Pu}^{239}$ ) and tritium ( $\text{H}^3$ ). Since  $\text{H}^3$  is a beta emitter and  $\text{Pu}^{239}$  an alpha emitter, it was advantageous to administer these elements simultaneously and to analyze for each element directly in the presence of the other without the necessity of carrying out any chemical separations.

## EXPERIMENTAL

*General Procedures*—The experiments described in this paper utilized 200 gram Sprague Dawley female rats and a growing mongrel dog. Experimental details which relate to the dog will be described later. The rats were kept individually in metabolism cages which were especially constructed to facilitate the daily collection of urine and feces separately. All injections

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were made by the intraperitoneal route. Eight rats were given a single injection of 20 ml of the  $\text{Pu}^{239}$  plus  $\text{Y}^{91}$  solution. Thirty minutes later three of the animals were administered 40 ml of the zirconium citrate solution (a total of 51.4 mg as  $\text{Zr}$ ) and two others were given 40 ml of a 5 per cent sodium citrate solution (early treatment). Of the remaining three control rats, one was given 40 ml of the zirconium citrate solution seventy-two hours after the administration of the radioelements (late treatment).

The rats were sacrificed six days after receiving the radioelements. The liver, spleen, kidneys, femurs, and carcass were analyzed separately.

**Radioelement Solution**—The radioelement solution was at a pH of 5.6 and had the following composition per milliliter: 40  $\mu\text{g}$  of  $+4 \text{Pu}^{239}$  ( $2.7 \times 10^6$  counts per minute under the counting conditions), 2.3  $\mu\text{c}$  of  $\text{Y}^{91}$  ( $1.3 \times 10^6$  counts per minute under the counting conditions), 2 per cent citric acid, 0.15M in  $\text{Na}^+$  and 0.05M in  $\text{Cl}^-$ .

**Preparation of the Zirconium Citrate Solution**—The zirconium was converted to a soluble citrate complex form. The solution of this complex salt is stable toward wide changes in temperature, pH, dilution, and to phosphate ion. A 100 ml batch is prepared as follows: To 18 ml of a filtered solution of zirconyl chloride ( $\text{ZrOCl}_2$ ) containing 130 to 140 mg as  $\text{Zr}$  per milliliter add, while stirring, 80 ml of a 12.5 per cent solution by weight of sodium citrate,  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ . A precipitate which first forms, presumably zirconium hydroxide, subsequently dissolves. Two milliliters of a 20 per cent solution of  $\text{NaOH}$  are added and the zirconium citrate solution is filtered through quantitative filter paper. The final solution is clear and colorless. It has a pH of  $\sim 6.3$  and contains about 25 mg as  $\text{Zr}$  per milliliter and the equivalent sodium and citric acid concentration of a 10 per cent sodium citrate solution. Because of its hypertonicity ( $\sim 1.1\text{M}$  in  $\text{Na}^+$ ) it is advisable to dilute the solution at least 1:1 with distilled water, as was done in the present experiment. For intravenous injection a fourfold dilution yielding a near isotonic solution and 6 mg as  $\text{Zr}$  per milliliter is preferable.

TABLE I EXCRETION OF ZIRCONIUM IN RATS FOLLOWING INTRAPERITONEAL ADMINISTRATION OF ZIRCONIUM CITRATE

DAYS ELAPSED AFTER FIRST ZP INJECTION	TOTAL MG AND PER CENT OF TOTAL INJECTED DOSE OF ZR							
	RATS INJECTED WITH 100 MG ZR ON 0 AND 3RD DAY				RATS INJECTED WITH 50 MG ZP ON 0 AND 1ST DAY			
	URINE		FECES		URINE		FECES	
	MG	%	MG	%	MG	%	MG	%
0-1	78	78	0.05	0.05	43.5	87	<0.05	<0.1
1-2	0.7	0.7	0.8	0.8	4.4	4.4	0.5	0.5
2-3	0.3	0.3	---	---	0.5	0.5	---	---
3-4	90	45	0.4	0.2	0.7	0.7	---	---
4-5	1.6	0.8	0.4	0.2	0.5	0.5	0.2	0.2
5-6	1.4	0.7	2.0	1.0	0.8	0.8	2.0	2.0
7-8	0.2	0.1	0.6	0.3	0.05	0.05	<0.05	<0.05
9-10	0.06	0.03	---	---	0.4	0.4	---	---
11-12	0.2	0.1	0.1	0.05	0.3	0.3	<0.05	<0.05
13-14	0.2	0.1	0.06	0.03	0.3	0.3	---	---
	173	87	$\sim 4.4$	$\sim 2.2$	91	91	$\sim 2.9$	$\sim 2.9$

The zirconium in this solution is put of in anion complex and the approximate formula of the chelated compound<sup>6</sup> formed is  $\text{Na} [\text{ZrO}(\text{C}_6\text{H}_5\text{O}_7)]$

*Excretion of Zirconium*—In a separate experiment the excretion rate of the zirconium citrate was studied (Table I). Within the first twenty four hours following the administration of zirconium citrate, the bulk of the dose is excreted in the urine. The analyses for zirconium were made by gravimetric method in which the phosphate of zirconium is precipitated from 10 per cent sulfuric acid.<sup>3</sup>

*Toxicity of Zirconium and Other Metals*—In rats the zirconium citrate salt appears to be innocuous and does not seem to result in chronic intoxication.<sup>4</sup> Large frequent doses as high as 1.5 Gm per kilogram as  $\text{Zr}$  did not cause any deaths. Investigations with a similar citrate salt of zirconium showed that the  $\text{LD}_{50}$  for acute toxic levels is 1.7 Gm as  $\text{Zr}$ .<sup>4</sup> As much as 3.6 Gm per kilogram of  $\text{Zr}$  is tolerated by rats when administered once daily in doses of 0.45 Gm per kilogram.

The possibility of using  $\text{La}$ ,  $\text{Ce}$  and  $\text{Cb}$  for metal displacement studies was tested. However, it was found that as little as 12 mg per kilogram of the chlorides of  $\text{La}$  and  $\text{Ce}$  proved fatal to rats within three days when given intravenously. Fifty milligrams of  $\text{Cb}$  per kilogram in the form of sodium columbate gave rise to symptoms of severe intoxication of a chronic nature.

*Methods of Analysis*—The excreted and weighed tissues were prepared for analysis by repeated ashing with concentrated  $\text{HNO}_3$  and 30 per cent  $\text{H}_2\text{O}$  until a white ash remained. The ash was dissolved in 3M  $\text{HNO}_3$  and the solution transferred to a volumetric flask and made up to volume. The urine samples obtained on the day following zirconium administration contained a considerable amount of insoluble oxides of zirconium. In such cases the solutions were well shaken before an aliquot for counting was taken so as to include a proportional amount of the suspended material.

The samples were assayed for  $\text{Y}^{91}$  activity by directly counting a dried aliquot through a thin mica end window Geiger tube. The samples for  $\text{Pu}^{239}$  activity were mounted on a  $1\frac{1}{2}$  inch platinum disc and the alpha activity was determined in a proportional  $\alpha$  counter. The dried sample weights were kept to a minimum in order to prevent significant losses through self absorption. Adjustments were made to insure that no  $\text{Y}^{91}$  beta activity was being recorded in the alpha counting circuit. The alpha or beta activity in a sample was periodically referred to a sample from the original injection solutions which were mounted and counted in an identical manner. Aliquots of the samples from the original injection solutions were obtained after making several dummy injections into a volumetric flask.

#### RESULTS OBTAINED WITH RATS

*Comparison of Normal  $\text{Pu}$  and  $\text{Y}$  Metabolism*—The fact that the  $\text{Pu}$  and  $\text{Y}$  were administered simultaneously affords a unique opportunity for directly comparing the metabolism and distribution of these elements under identical circumstances.

<sup>6</sup> The chelate nature of the compound and its probable formula is based on information communicated by Dr. Loren C. Hurd of the Rohm and Haas Company, Philadelphia, Pa. Actually the salt is sodium zirconyl citrate but for our purposes it is convenient to refer to it as zirconium citrate.



TABLE II. EFFECT OF ZIRCONIUM AND SODIUM CITRATE ON THE DISTRIBUTION AND EXCRETION OF YTTRIUM AND PLUTONIUM IN RATS\*

TISSUE	PER CENT OF INFECTED DOSE PER TOTAL TISSUE						PER CENT OF INFECTED DOSE PER GRAM FRESH TISSUE											
	UNTREATED CONTROLS			FARIY ZIRCONIUM TREATED			IARIY CITRATE TREATED			UNTREATED CONTROLS			IARIY CITRATE TREATED			FARIY ZIRCONIUM TREATED		
	Y		Pu	Y		Pu	Y		Pu	Y		Pu	Y		Pu	Y		Pu
	Y	Pu		Y	Pu		Y	Pu		Y	Pu		Y	Pu		Y	Pu	
Urine	27.0	1.41		3.3	2.68		6.37	51.3		---	---	---	---	---	---	---	---	---
	$\pm 3.2$	$\pm 0.7$		$\pm 0.7$	$\pm 0.2$		$\pm 4.3$	$\pm 2.2$		---	---	---	---	---	---	---	---	---
Liver	2.9	1.8		5.2	7.5		5.8	4.5		---	---	---	---	---	---	---	---	---
	$\pm 0.3$	$\pm 1.7$		$\pm 1.2$	$\pm 0.9$		$\pm 0.9$	$\pm 0.5$		---	---	---	---	---	---	---	---	---
Thymus	4.2	7.29		5.6	8.03		1.6	1.20		2.9	4.90		3.8	5.55		1.06	0.82	
	$\pm 0.3$	$\pm 0.06$		$\pm 0.3$	$\pm 0.8$		$\pm 0.1$	$\pm 0.05$		0.14	1.14		0.09	1.71		0.18	1.06	
Kidney	1.5	11.9		0.93	14.0		1.8	10.7		1.25	0.56		0.64	0.45		0.66	0.67	
	$\pm 0.4$	$\pm 1.3$		$\pm 0$	$\pm 0$		$\pm 0.2$	$\pm 2.0$		0.47	0.51		0.40	0.65		0.51	0.39	
Spleen	0.7	0.96		0.99	0.72		1.1	1.14		0.34	0.40		0.30	0.34		0.14	0.17	
	$\pm 0.7$	$\pm 0.13$		$\pm 0.14$	$\pm 0.11$		$\pm 0.2$	$\pm 0.30$										
Carcass	0.70	0.33		0.26	0.42		0.33	0.25										
	$\pm 0.05$	$\pm 0.01$		$\pm 0.01$	$\pm 0.7$		$\pm 0.06$	$\pm 0.03$										
	61.9	72.4		53.7	62.6		25.7	30.9										
	$\pm 1.2$	$\pm 0.7$		$\pm 1.1$	$\pm 0.2$		$\pm 3.2$	$\pm 2.5$										

\*Values equated to 100 per cent (actual recoveries averaged 89 plus-minus 5 per cent).

Each value is an average of results obtained from two rats for the untreated controls, two rats for the sodium citrate treated three rats for the early zirconium citrate treated and one rat for the late zirconium citrate treated.

The sodium citrate treated rats received 1 ml of a 5 per cent solution one half hour after the late zirconium citrate solution of  $Y^{91}$  plus  $Pu^{239}$  while the early zirconium treated rats received 51.1 mg as Zn one-half hour after the  $Y^{91}$  plus  $Pu^{239}$  solution. The late zirconium treated rat received 51.1 mg as Zn three days after the  $Y^{91}$  plus  $Pu^{239}$  solution. All injections were made by the intraperitoneal route.

Considerable differences in their behavior were shown especially in the urinary excretion and retention by the liver (Table II)

During the six day period the rats excreted in the urine nearly twenty times as much Y as Pu (27 per cent and 14 per cent respectively). On the other hand, the fecal output of Pu was about twofold greater than the fecal

### DISTRIBUTION AND EXCRETION OF PLUTONIUM ( $Pu^{239}$ ) IN UNTREATED AND TREATED RATS

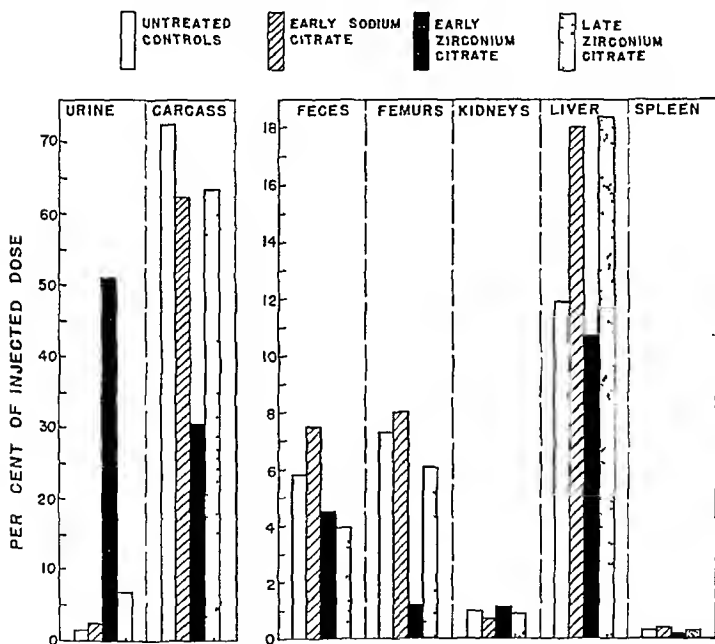


Fig 1—Effect of zirconium and sodium citrate treatment on the distribution and excretion of Pu (IV) in rats six days after the intraperitoneal administration of the radioelement.

excretion of Y. The ratio of urinary to fecal excretion one day after injection was about 20 for Y and about 0.5 for Pu. At the sixth day after injection the ratio was approximately 1 for Y and 0.04 for Pu. These ratios emphasize the differences in the nature of the excretion between these two elements.

The kidney retained more than twice as much Y as Pu while the liver retained about eight times more Pu (11.9 per cent and 1.5 per cent respectively). The deposition of Pu in the skeleton was 1.7 times as great as that of Y as

# DISTRIBUTION AND EXCRETION OF YTTRIUM ( $Y^{91}$ ) IN UNTREATED AND TREATED RATS

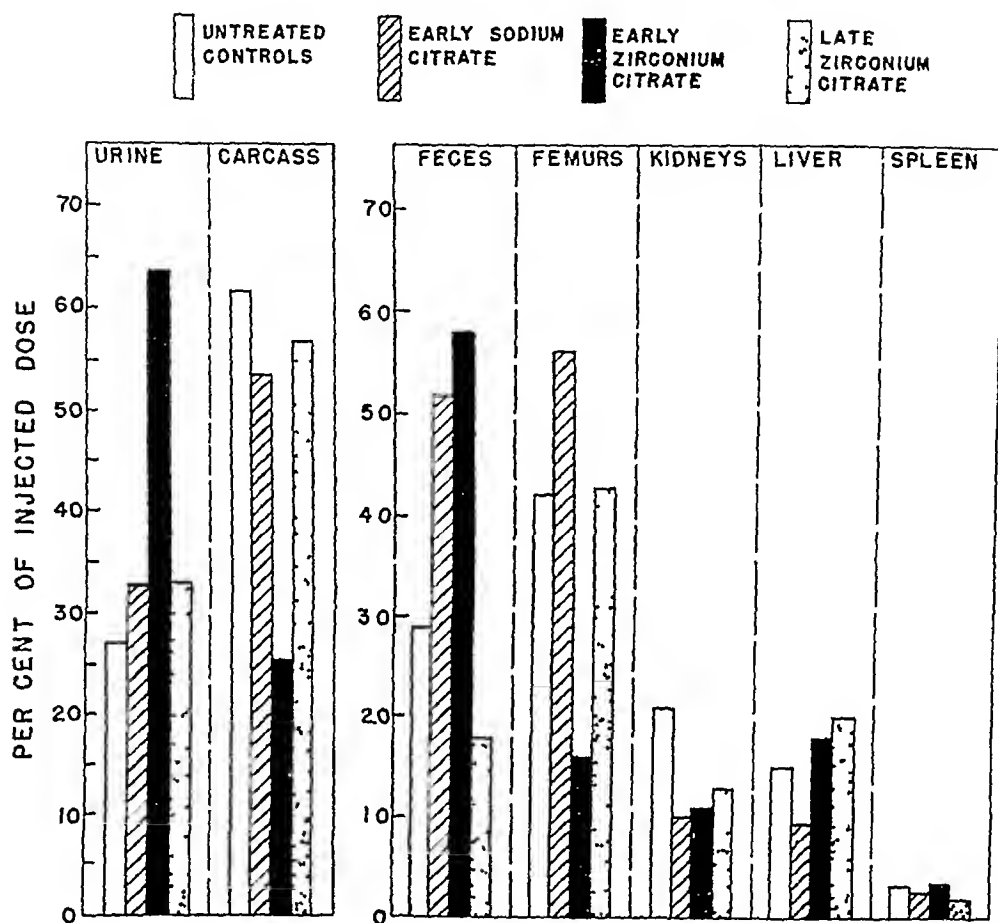


Fig 2—Effect of zirconium and sodium citrate treatment on the distribution and excretion of  $Y^{91}$  in rats six days after the intraperitoneal administration of the radioelement.

indicated by the femur content. No significant differences in the amounts deposited in the spleen were found. The carcass, which included the pelt, muscle, blood, skeleton, and gastrointestinal tract retained 72 per cent of Pu and 62 per cent of the Y. With respect to Pu the results obtained are in good quantitative agreement with those reported by Cairnt and co-workers.<sup>6</sup>

The differences in excretion and distribution between Pu and Y are probably indicative of the greater tendency of Pu to become colloidal in the physiologic pH region. This point is discussed later in the paper.

*Effect of Treatment on the Urinary and Fecal Excretion of Pu and Y*—The over-all distribution and excretion of plutonium and yttrium in treated and untreated rats at the end of the six-day period are summarized in Table II and presented graphically in Figs 1 and 2. The daily excretion levels are shown in Figs 3, 4, and 5. During the six-day period the rats lost about 5 per cent of their body weight.

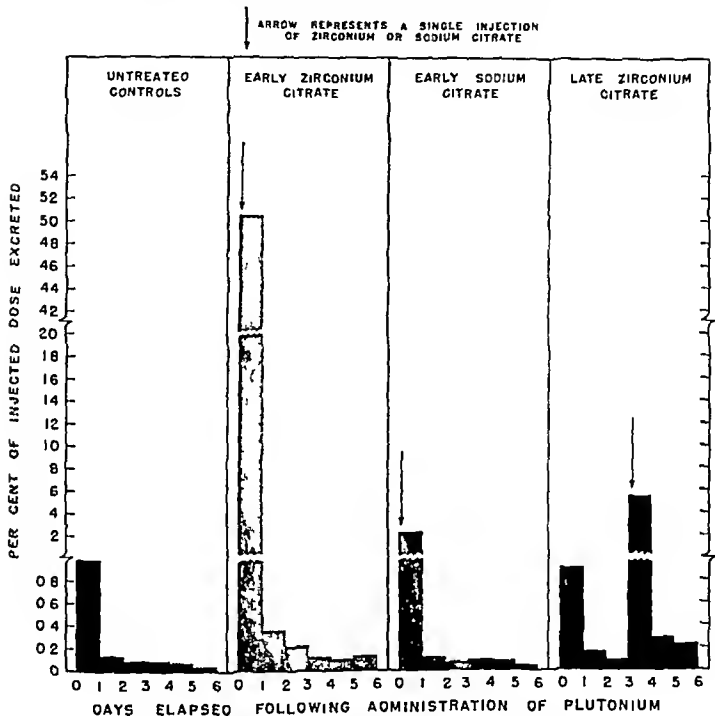
EFFECT OF ZIRCONIUM AND SODIUM CITRATE TREATMENT ON THE DAILY URINARY EXCRETION OF  $Pu^{239}$ 

Fig 3—See text for information regarding the injections

Following a single injection of 51.4 mg of Zr the urinary excretion of Pu in the early treated rats was increased from about 1 per cent of the total injected dose to about 50 per cent, a factor of 50. At the same time the urinary excretion of Y was increased from about 25 per cent to 60 per cent, or nearly two and one half fold. Of interest is the fact that the urinary excretion of Pu (and to a lesser extent of Y) continued to be appreciably greater than that of the control animals for the succeeding five days (Figs 3 and 4). The sustained nature of the heightened urinary excretion of Pu confirms earlier findings.<sup>2</sup> When a single injection of 51.4 mg of Zr was administered three days after the Pu and Y, the urinary excretion of Pu was still increased to a remarkable extent, namely from 0.08 per cent to more than 5 per cent, a factor of more than 60. Similarly, the excretion of

# EFFECT OF ZIRCONIUM AND SODIUM CITRATE TREATMENT ON THE DAILY URINARY EXCRETION OF $Y^{91}$

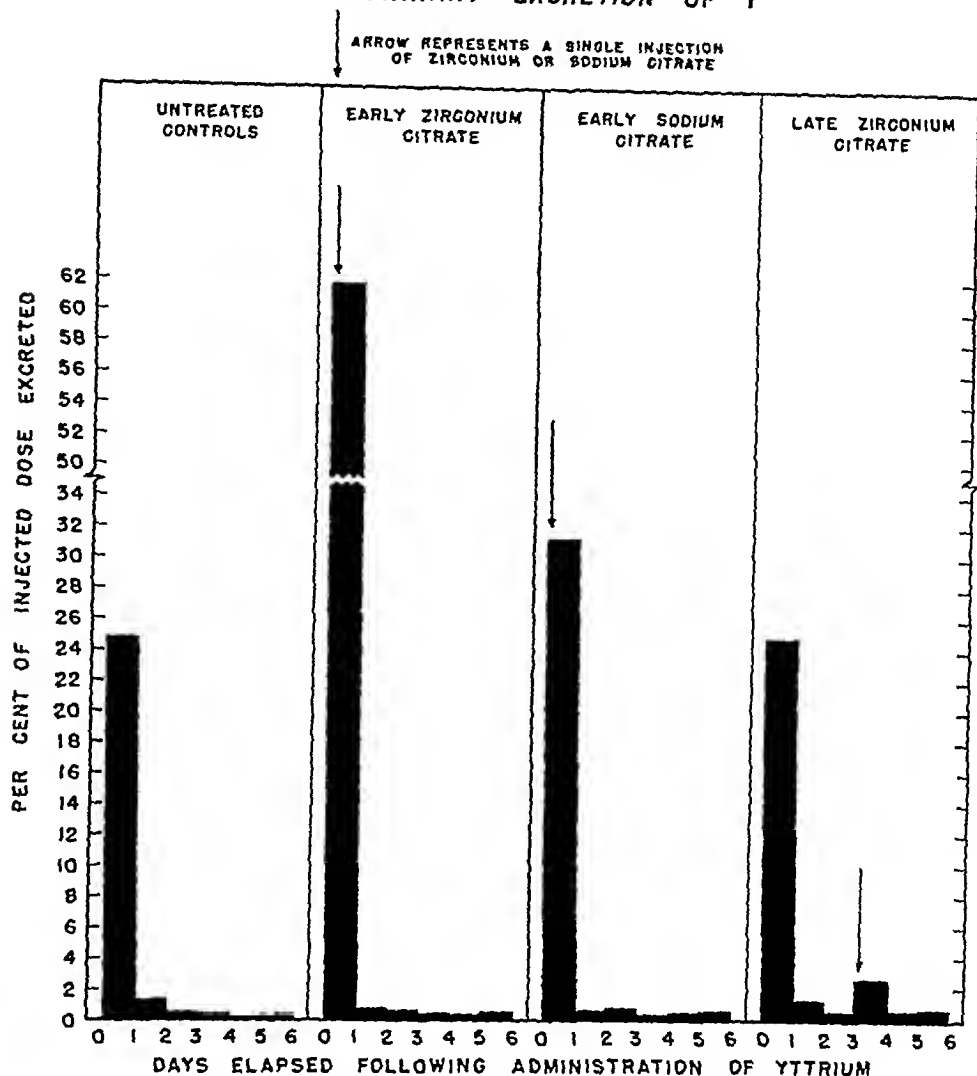


Fig 4—See text for information regarding the injections

$Y$  was increased about tenfold, viz 0.3 per cent to  $2\frac{1}{2}$  per cent. Sodium citrate did not appear to significantly change the urinary excretion of Pu or  $Y$ .

The fecal excretion of Pu or  $Y$  was little affected by zirconium or sodium citrate administration. In the case of  $Y$  early treatment increased fecal excretion by a factor of 2 over the six-day period while that of Pu was not significantly changed (Fig 5).

*Effect of Treatment on the Body Distribution of Pu and  $Y$* —The amount of Pu and  $Y$  deposited in the skeleton was remarkably reduced following the early administration of a single injection of zirconium citrate. The Pu content in the femurs decreased from 7.3 per cent to 1.2 per cent, a reduction of

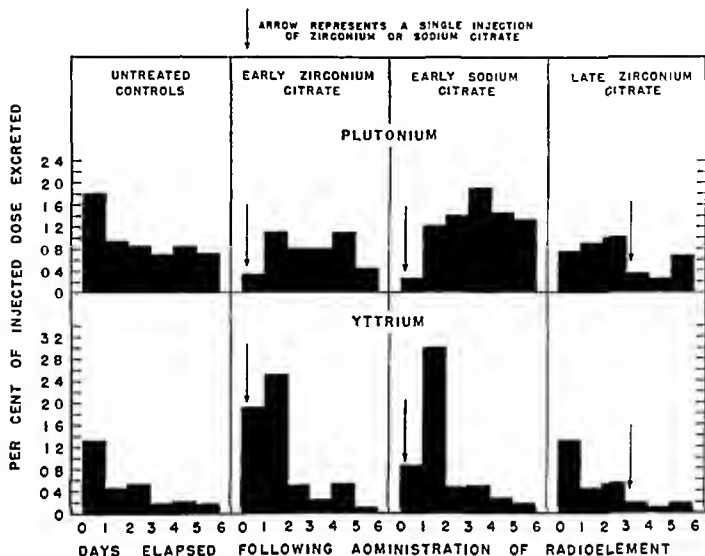
EFFECT OF ZIRCONIUM AND SODIUM CITRATE TREATMENT ON THE DAILY FECAL EXCRETION OF  $\text{Pu}^{239}$  AND  $\text{Y}^{91}$ 

Fig 5—See text for information regarding the injections

sixfold, while that of Y was decreased from 42 per cent to 16 per cent a factor of 2.6. In the rats treated with Zr three days after exposure, a small decrease in skeletal Pu but not of skeletal Y was observed. The amounts of Pu and Y retained by the carcass in the early treated rats were reduced by a factor of about 2.3.

No significant changes in the amounts of Pu and Y deposited in the spleen, liver, or kidneys were found following administration of zirconium citrate. In a previous experiment it was found that the amount of Pu deposited in the liver was reduced by a factor of 2 to 3 following Zr administration. However, the fact that the animals in the previous experiment were sacrificed eighteen days after the administration of Pu and Zr may be responsible for the difference. It is of interest to add that in the previously reported experiments no change in the amount of Pu deposited in the lungs was noted following administration of zirconium citrate.

Hamilton and co workers<sup>6</sup> have carried out experiments on the effect of zirconium citrate on the distribution and excretion of rats injected intramuscularly or intravenously with plutonium or yttrium. The results of these experiments are in good agreement with those reported here.

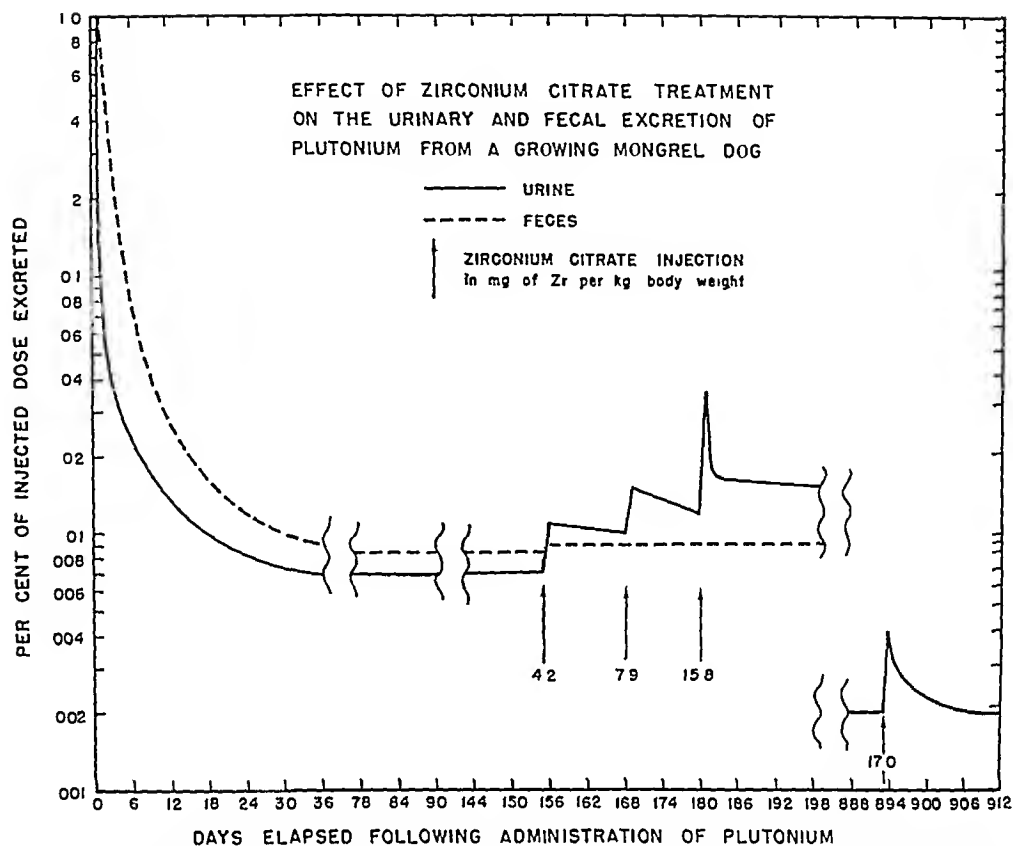


Fig 6—Effect of zirconium citrate on the rate of excretion of Pu by a growing dog following intravenous administration of 0.423 mg of Pu (IV). The zirconium citrate was given intravenously. The dog weighed 7.3 kg when injected, 11.5 kg 150 days after the Pu injection and 12 kg after 890 days.

#### RESULTS OBTAINED WITH A GROWING DOG

A growing mongrel dog weighing 7.27 kilograms was injected intravenously on Oct 25, 1945, with 0.423 mg of Pu (0.058 mg per kilogram) in the form of  $\text{Pu}(\text{IV})$  nitrate. The rate of urinary and fecal excretion of Pu was quite rapid during the subsequent two weeks and then reached a nearly constant level about thirty days after the administration of Pu (Fig 6). During the two weeks immediately following the injection of Pu the dog excreted 1.1 per cent of the injected dose in the urine and 2.7 per cent in the feces, or a total of 3.8 per cent. On the thirtieth day after the injection the average urinary excretion of Pu was 0.007 per cent of the injected dose. The average daily fecal excretion of Pu was about 0.0085 per cent and the ratio of average urinary to fecal excretion was 0.8. This ratio is in substantial agreement with data obtained with human subjects at thirty days.<sup>6</sup> Four months after injection the urinary and fecal excretion of Pu was essentially unchanged from the thirty-day value. It is of interest to note that at this time the concentration of Pu per milliliter of circulating blood was  $0.025 \mu\text{e}$  or  $10^{-4}$  per cent of the injected dose.

Five months after the dog received Pu, intravenous administration of small amounts of zirconium citrate was begun. At this time the dog's weight was 11.5 kilograms. An injection of only 4.15 mg per kilogram of Zr was followed by a definite increase in the urinary excretion of Pu (Fig. 6). Subsequent larger doses of zirconium citrate indicated that the extent of the increase in the excretion level of Pu was proportional to the amount of Zr injected. The increase in the excretion level was greatest during the few days immediately following the administration of zirconium citrate. However, the increased rate of excretion was continued for at least two to three weeks after cessation of Zr treatment. Following injections of 4.15, 7.85, and 15.8 mg per kilogram of Zr the average per cent increase in Pu excretion on the tenth day after each Zr injection was 35, 75, and 165 per cent respectively. Inasmuch as the dosage levels of Zr were considerably less than the maximal dose, probably by a factor of at least 10 to 100, it appears that the biologic half-life of Pu in the body can be reduced to a significant extent even in cases of chronic poisoning. The fact that the treated dog had undergone a 60 per cent increase in body weight by the time the zirconium citrate was administered speaks well for the effectiveness of the treatment because the burying or overlying of the deposited Pu by newly laid tissue would probably tend to decrease the displacing action of Zr.

Nearly two and one half years after the injection of Pu the urinary excretion level had dropped to 0.002 per cent of the injected dose. This is probably lower than would be the case if the dog had reached its full growth before receiving plutonium for the above mentioned reason. It is of interest to note that injection of 17 mg per kilogram of Zr at this late date was followed by a twofold increase in the urinary excretion of Pu.

The fecal excretion of Pu was scarcely affected by the administration of zirconium citrate.

#### DISCUSSION AND GENERAL THEORETICAL CONSIDERATIONS

The distribution and excretion of metals are, in many instances, greatly dependent upon the route of administration, the chemical state of the metal, and the solvent. With metals possessing colloidal tendencies these factors are even more critical. The effectiveness of zirconium citrate treatment for any one metallic radioelement may vary considerably, particularly with those having colloidal tendencies. In interpreting the behavior of cationic elements in the body it is necessary to consider their chemical properties very closely, especially those relating to the formation of hydroxides, soluble complex ions, and insoluble compounds.

A radioelement is bound to various tissues by chemical or physical forces or a combination of the two depending on the environment. The displacement process is essentially an exchange reaction. When the exchange reaction is ionic in nature then the displacing property of a cation is dependent on its concentration, valence and hydrated radius, and bodily distribution. The higher the valence and concentration and the smaller the hydrated radius



the greater is the displacing ability. The ideal cation should combine maximum displacing properties with a minimum toxicity. Furthermore, its displacing action should be sustained for long periods. Thus a metallic cation which is not destroyed by metabolic processes would be preferable to metabolizable substances such as citrates and acetates.

Some cationic radioelements are bound to surfaces by insoluble compound formation, as, for example, with bone phosphate. In a physical chemical system, and presumably under biologic conditions, elements bound in such a manner can be displaced by cations which form similar insoluble compounds, the degree of displacement being dependent upon the solubility and concentration of the displacing cation relative to the adsorbed cation. Complexing agents facilitate the removal process.

Colloids and suspended matter are often irreversibly adsorbed by surfaces. In a biologic system the only safe manner for effective removal of such substances is through the solubilization action of complexing agents. The slight action of sodium citrate in elevating the urinary excretion of Pu and Y is probably due to the complexing action of the citrate ion on the otherwise undiffusible fractions of these radioelements. A large part of the Pu in blood and urine is not ultrafilterable. However, the addition of 0.001M citric acid is followed by a marked increase in the diffusible fraction of Pu, that is, the form in which it is available for excretion through the membranes of the kidney and bowel. A discussion of the action of citrate ion under physiologic conditions, and with particular reference to lead poisoning, is given by Kety.<sup>8</sup> The use of complexing agents such as citrates may be of supplementary value for removing those radioelements with which they form very stable complex ions.

The deposition of the alkaline earths in the bone differs markedly from that of plutonium, yttrium, and zirconium. Thus, radiostrontium is deposited mainly in the mineral structure of the bone while Pu, Y, and Zr are concentrated in the osteoid matrix.<sup>1</sup> It was not expected, therefore, that zirconium citrate would exert an appreciable displacing action on strontium in view of its marked metabolic and chemical differences. In experiments which will be reported at a later date it was found that no significant increases in the urinary or fecal excretion of Sr were observed and neither was its concentration in the liver or skeleton changed as the result of zirconium or sodium citrate administration.

The most effective procedure for hastening the removal of mobile deposits of plutonium or yttrium from the body would be to administer zirconium citrate at least twice daily over an extended period. The sooner treatment is begun and the larger the initial dose, the greater should be the net reduction in body content, particularly in the amount deposited in the skeleton. The effect of oral administration of zirconium citrate is under investigation.

It is hoped that a correlation of the results of these and subsequent metal displacement experiments with the chemical and metabolic properties of the radioelements involved will facilitate the devising of effective procedures for removing radioelements from the body.

## SUMMARY

A study was made of the effect of a single dose of zirconium citrate on the excretion and distribution of plutonium and radioyttrium in rats and a growing dog. It was found that early administration of zirconium citrate to rats increased the excretion of Pu from 1 per cent to 50 per cent and that of Y from 25 per cent to 60 per cent of the injected dose during a twenty four hour period. The amount of Pu and Y deposited in the skeleton was reduced by factors of 6 and 2.6 respectively. No significant changes in the Pu or Y contents of the feces, liver, spleen or kidney were found.

The administration of zirconium citrate to a dog five months and two and one half years after exposure of Pu was followed by a sustained increase in the excretion rate. The extent of the increase was proportional to the dose of zirconium citrate.

A discussion is given of the chemical concepts involved in applying metal displacement principles to the removal of radioactive metals from the body.

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# THE SIGNIFICANCE OF THE AMINO ACID COMPOSITION OF THE PROTEINS EXCRETED BY THE NEPHROTIC CHILD

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THE view that the nephrotic syndrome is the result of a general metabolic disorder is not as yet adequately supported by the evidence, but there is no question among clinicians that the problem of managing the disease is a metabolic one<sup>1</sup>. The problem stated in its simplest terms is to achieve a compensatory repletion of proteins lost to the organism by excretion into the urine and abdominal fluid. Although the solution of this problem may or may not in itself correct the primary cause of the disease, it may accomplish a favorable prognosis by mitigating the hazards of the concomitant malnutrition. To realize the magnitude of the cachexia induced by this disease, it is only necessary to recall that on the basis of Rubner's values<sup>2</sup> each gram of protein lost via the urine alone is equivalent to a loss of 5.3 Gm. of body tissue—and a daily output of 25 Gm. of urinary protein is not uncommon in nephrotic children.

In cognizance of this aspect of the disease a variety of proteins and protein products have been empirically assayed orally and parenterally in the therapy of nephrosis with varying degrees of success<sup>3, 4, 5</sup>. These efforts have attempted primarily to replenish protein losses by augmenting protein input over and above the amount which can be given in the form of normal foods. Since a similarity in amino acid patterns of the proteins administered and those lost in the biologic fluids during the course of the disease might reasonably afford a greater measure of compensation than those of dissimilar pattern. We have undertaken to obtain and report data, heretofore lacking, on the amino acid composition of proteins excreted by children afflicted with the nephrotic syndrome. In addition to furnishing a guide as to the types of proteins most suitable for dietotherapeutic treatment, these results disclose (a) that the composition of the urinary proteins varies with the severity of the symptoms, (b) that the composition of the proteins found in the abdominal fluid may or may not simulate that of the proteins occurring simultaneously in the urine, and (c) that only in the improved stages of the disease does the albumin of the urine approximate the albumin of the blood in amino acid composition.

## EXPERIMENTAL

Since we were concerned primarily with determining the total quantity of amino acids lost in the excreted proteins as a whole rather than the variety of protein species

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it was considered expedient to sub-fractionate the proteins and to forego the niceties of electrophoretic separation? It seems reasonable to assume that for diatherapeutic interpretations the over-all amino acid pattern of the urinary proteins irrespective of kind, is the practical criterion. By the same token we shall not attempt a detailed classification of the nature of the kidney involvement. Considerable diversity of clinical opinion prevailed in several of the cases reported here regarding the extent of glomerular damage which coexisted with the nephrotic symptoms. Differentiation on the basis of urea clearance could not be made because of the inconsistency of these data. For our purpose the chemical data on the blood and urine provide objective and adequate criteria.

*Isolation and Analysis of Proteins of Nephrotic Patients*.—In principle the techniques described by Miller and co-workers<sup>8</sup> have been employed for the separation of the urinary and abdominal proteins and their experiences on the relation of the nature of protein mixtures excreted and severity of the disease in the adult have been found to apply for the child.

The urine of the patient was collected and pooled for periods which were considered "critical" or "improved" phases by chemical measurements and clinical observations. During critical period (e.g., the period during which marked nephrotic symptoms were present) the albumin:globulin ratio of the urinary proteins was uniformly below 3.00. During the period of improvement (subsiding of nephrotic symptoms, disappearance of edema, etc.) the albumin:globulin ratio was found to be 10.0 or above.

To each daily collection of urine was added an equal volume of saturated ammonium sulfate, and the collections were pooled for each period usually about five days. The clear supernatant liquid was siphoned off into another jar and to this was added 10 per cent of its volume of 50 per cent trichloroacetic acid to precipitate the albumin. The precipitate from the ammonium sulfate treatment (globulin fraction) was washed three times by decantation. The precipitate (albumin) from the trichloroacetic acid treatment was washed three times in the same manner with 5 per cent trichloroacetic acid. After washing the suspended proteins were poured into the middle compartment of a three-chambered electrolytic cell<sup>9</sup> and dialyzed to remove inorganic impurities. After dialysis each specimen was collected in a 250 cc. centrifuge cup and washed by dispersion in water and dried by successive suspension in 50 per cent, 75 per cent and absolute acetone centrifuging and decanting after each washing. The protein was dried overnight in the 50° oven ground to a fine powder, and stored in small tightly stoppered bottles.

In some urines of the improved period a precipitate was obtained after treatment with ammonium sulfate as described which was found not to be globulin but almost pure urea acid. The urines contained albumin.

From the individual protein samples so prepared, 100 mg. portions were removed for moisture and ash determinations; two 25 mg. aliquots were removed for micro-Kjeldahl determinations; a 50.0 mg. portion was hydrolyzed with 10 per cent NaOH for the tryptophane determination, and a 2 Gm. portion was hydrolyzed with 6N HCl in the usual manner for the determination of other amino acids.

The proteins of the abdominal fluids were prepared and analyzed in the same manner as the urinary proteins.

## RESULTS

In Table I are collected the amino acid data on the urinary proteins of four children who had a long history of nephrosis and died of the disease after these studies were done.

Examination of the analytical results discloses that on the basis of amino acid content the urinary albumin and globulin are distinct protein fractions. However, the urinary albumin and globulin from patients Sm and Te which occurred in nearly the same albumin:globulin ratio are somewhat similar in amino acid composition, whereas the protein fractions from patient Dd which were derived from urines of a lower albumin:globulin ratio contain more

TABLE I ANALYSIS OF URINARY PROTEINS OF NEPHROTIC PATIENTS WHO DIED  
(The amino acid values are given as per cent amino acid N of total N)

PATIENT, SEX, AND AGE AT ONSET OF NEPHROSIS	SM, FEMALE, 3 5 YR		TC, MALE, 20 YR		DD, FEMALE, 40 YR		AS, MALE, 18 YR					
	Critical	76	Critical	41	Critical	51	Critical I	22	Improved	Critical II	Critical III	
Clinical condition												
Time from onset of nephrotic symptoms (wk)												
Blood constituents												
Cholesterol (mg %)	667		571		640		802		287	726	700	
Amino N (mg %)	7 71		6 75		7 27		7 60		-	-	-	
Nonprotein N (mg %)	135		60		64		51		28	-	100	
Hemoglobin (Gm %)	6 10		9 30		9 50		8 10		8 60	-	-	
Total plasma proteins (Gm %)	3 76		3 85		4 64		4 20		5 20	3 70	3 60	
A G ratio	0 28		0 33		0 21		0 10		1 28	0 32	0 34	
Urinary proteins												
Average daily output (Gm)	13 0		14 7		14 2		14 6		0 50	7 70	19 6	
Total proteins (Gm %)	1 12		1 21		1 33		3 10		0 20	1 60	23 0	
Average A G ratio	2 73		2 65		1 68		1 73		∞	1 73	-	
N content (corr) (%)	ALBU MIN	GLOB ULIN	ALBU MIN	GLOB ULIN	ALBU MIN	GLOB ULIN	ALBU MIN	GLOB ULIN	ALBU MIN	GLOB ULIN	ALBU MINOID	
Moisture (%)	13 7	15 4	13 6	15 4	13 7	14 0	13 6	13 8	14 5	13 3	15 7	
Ash (%)	5 8	3 2	4 5	4 0	3 2	4 0	8 6	4 6	6 4	4 2	4 2	
Hydrolysates	0 6	0 9	0 2	0 5	0 2	0 3	0 3	0 2	0 4	0 3	0 3	
Amino N <sup>10</sup> *	81 2	79 2	73 3	70 8	78 0	78 0	77 4	80 6	71 2	79 4	80 0	
Arginine <sup>11</sup>	11 2	11 2	11 8	11 8	12 7	13 8	12 8	13 9	10 4	12 2	13 2	
Ileutidine <sup>12</sup>	6 9	5 3	6 5	5 2	6 2	6 9	6 7	6 9	6 4	6 5	6 9	
Methionine <sup>13</sup>	1 7	2 3	1 8	2 0	2 0	2 7	2 0	2 9	2 1	2 0	2 0	
Cysteine <sup>14</sup>	3 5	2 5	3 6	2 7	4 6	3 5	5 2	3 5	4 8	4 3	4 2	
Phenylalanine <sup>15</sup>	4 5	3 2	4 3	2 9	3 9	4 3	4 1	4 3	2 4	4 7	3 6	
Tyrosine <sup>16</sup>	2 0	2 2	1 8	2 1	2 1	2 1	2 1	2 1	2 0	2 2	2 1	
Isoleucine <sup>17</sup>	2 6	4 4	2 8	3 8	2 4	4 0	2 4	4 4	2 0	3 0	2 9	
β hydroxy amino N <sup>18</sup>	3 8	6 5	3 4	6 1	4 6	6 9	4 8	7 2	1 0	4 4	9 2	
Tryptophane <sup>19</sup>	1 9	2 5	1 8	2 3	1 9	3 0	1 8	3 0	1 5	2 0	3 1	

\*The figures represent bibliographic references

arginine,  $\beta$  hydroxy  $\alpha$  amino nitrogen and tryptophane. The possibility that the excretion of different albumins and globulins as indicated by their amino acid composition predominates at different stages of the disease is further indicated by the data obtained by the analyses of the urinary proteins of patient As who passed through an "improved" period before his death. The accord of the amino acid pattern of the urinary albumin and globulin of "critical" periods I and II of this patient and those of patient Dd which were obtained from urines having approximately the same albumin globulin ratios is surprising.

Following critical period I as indicated by chemical studies and clinical observations, the condition of patient As improved either spontaneously or because of the administration of 50 to 60 Gm daily of an enzymatic digest of lactalbumin (Edman, Sheffield). The analyses of the urinary proteins during this "improved" period disclosed the absence of globulin and the presence of an albumin fraction quite different in amino acid composition from the albumin of the patient isolated from the urine of critical period I. The downward shifts in the arginine, phenylalanine, and  $\beta$  hydroxy  $\alpha$  amino nitrogen are the most significant. Subsequently the patient relapsed, into critical period II, despite the continued administration of Edman, and, as noted, the composition of the urinary proteins returned to that of the proteins

TABLE II ANALYSES OF URINARY PROTEINS OF NEPHROTIC PATIENTS WHO RECOVERED  
(The amino acid values are given as per cent amino acid N of total N)

PATIENT, SEX, AND AGE AT ONSET OF NEPHROSIS	M, MALE 22 YR		MC MALE 20 YR			
Clinical condition	Critical	Improved	Improved			
Time from onset of nephrotic symptoms (wk)	25	30	92			
Blood constituents						
Cholesterol (mg %)	980	377	247			
Amino N (mg %)	81	72	78			
Nonprotein N (mg %)	42	30	32			
Hemoglobin (Gm %)	75	98	119			
Total plasma protein (Gm %)	42	59	66			
A/G ratio	0.3	1.1	1.0			
Urinary proteins						
Average daily output (Gm)	35	15	0.9			
Total proteins (Gm %)	0.8	0.4	0.4			
Average A/G ratio	3.3	10.5	12.0			
	ALBUMIN	GLOBULIN	ALBUMIN	GLOBULIN	ALBUMIN	GLOBULIN
N content (corr) (%)	13.3	13.7	13.5		13.9	
Moisture (%)	8.6	5.1	8.2		6.6	
Ash (%)	0.6	0.5	0.4	Only	0.3	Only
Hydrolysates						
Amino N	79.3	74.8	72.2		76.8	
Arginine	11.6	11.7	10.3		9.6	
Histidine	6.6	5.4	6.3		6.0	
Methionine	1.7	2.5	1.9	traces	1.8	traces
Cystine	5.5	2.6	4.7		4.6	
Phenylalanine	4.5	3.2	3.9		2.4	
Tyrosine	2.2	2.4	2.0		1.8	
Isoleucine	2.3	3.6	2.1	present	1.6	present
$\beta$ hydroxy $\alpha$ amino N	3.9	6.2	0.7		0.7	
Tryptophane	1.8	2.6	1.5		1.5	

of the critical period I. However, during the course of making urine collections for critical period II, a twenty-four hour urine collection was found to form a heavy white precipitate. This was removed by centrifugation and found to consist of almost pure protein, whereas the supernatant urine was free of protein. Solubility tests showed the substance to be insoluble in water, 10 per cent NaCl, and 10 per cent HCl, but readily soluble in 10 per cent NaOH. It could be readily precipitated from the alkaline solution by the addition of 20 per cent trichloroacetic acid or saturated ammonium sulfate. On the basis of these solubility characteristics this protein would appear to be an albuminoid<sup>20</sup>. This single specimen was purified by electrodialysis and the analytical results were listed under critical period III, the collection for critical period II was naturally terminated with the preceding twenty-four hour collection. Subsequently the condition of the patient rapidly became worse so that further twenty-four hour collections could not be made. Fractional collections showed no evidence of this protein. The analyses of this albuminoid showed it to be quite similar to the globulin of critical period II with the exception of the lower isoleucine and higher  $\beta$ -hydroxy  $\alpha$ -amino nitrogen values.

In Table II are collected the data on two patients who recovered from the disease and are at this writing, two years later, alive and well. It is to be noted that the critical period urinary proteins of patient Al which were isolated from urines having an average albumin globulin ratio of 3.3 are not unlike those of Sm and Te (Table I) which were obtained from urine having albumin globulin ratios of 2.73 and 2.65 respectively. The albumin secured from the urine of this patient on his improvement and that of patient Me (Table II) whose critical period collections were lost are to be compared with

TABLE III COMPOSITION OF ASCITIC FLUID PROTEINS OF NEPHROTIC PATIENTS  
(The amino acid values are given as per cent amino acid N of total N)

PATIENT, SEX, AND AGE AT ONSET OF NEPHROSIS	DD, FEMALE, 40 YR		AS, MALE, 18 YR	
Clinical condition	Critical		Critical II	
Fluid volume (cc)	4,500		4,420	
Total proteins (Gm %)	0.15		0.47	
A/G ratio	1.13		1.61	
	ALBUMIN	GLOBULIN	ALBUMIN	GLOBULIN
Nitrogen content (corr) (%)	14.1	14.1	13.5	12.9
Moisture (%)	8.9	9.6	3.1	2.8
Ash (%)	0.7	0.9	0.8	0.8
Hydrolysates				
Amino N	79.2	74.8	68.4	71.1
Arginine	12.1	9.9	12.7	12.9
Histidine	6.6	4.9	6.5	6.9
Methionine	0.7	2.3	1.9	1.8
Cystine	3.3	1.6	4.1	3.4
Phenylalanine	5.7	4.8	4.5	3.6
Tyrosine	2.5	2.3	2.3	2.3
$\beta$ hydroxy $\alpha$ amino N	2.4	7.5	4.5	7.2
Tryptophane	2.2	3.3	2.1	3.0

the albumin of patient As (Table I) during the improved period. It will be observed that the amino acid patterns of these three different protein specimens are in remarkable coincidence. The arginine, phenylalanine,  $\beta$ -hydroxy- $\alpha$ -amino nitrogen and tryptophane content of these albumin fractions is significantly and uniformly lower than that of the albumin fractions collected during the critical periods. Indeed the amino acid composition of this albumin simulates that of serum albumin. These patients also were given 50 to 60 gm of L-dum daily and their recovery may or may not have been influenced by this therapy.

In order to more completely assess the protein losses during nephrosis we also analyzed the ascitic fluid secured by abdominal tapping of two of the patients coincidentally with the designated urine collection periods. Scrutiny of these analytical results (Table III) discloses that the albumin and globulin of the ascitic fluid of Dd which has only one tenth the total protein concentration as the corresponding urine are not only present in a different albumin globulin ratio from that of the urinary proteins (Table I) but also as might be expected from the foregoing findings possess a vastly different amino acid configuration. The ascitic fluid from As which has about one third the protein concentration as the corresponding urine pool contains proteins which on the basis of amino acid composition are quite similar to those of the urine and as might be anticipated, the albumin and globulin ratios of the two fluids are in good accord. These observations suggest that the species of proteins which filter into the abdominal cavity are not necessarily identical with those filtering into the urine. The quantitative and qualitative disparity or similarity of the proteins occurring in these fluids probably reflects the nature and extent of the existing kidney damage.

It is obvious that the validity of the deductions made regarding the similitude or dissimilitude of the proteins described heretofore is seriously limited by the lack of data on the amino acids for which no quantitative measurements were made. This deficiency arose primarily from the lack of adequate chemical methods. However we were able by means of paper chromatograms<sup>1</sup> to ascertain, within the limits of this technique, the general distribution of the other amino acids. Thus it was found that the lysine content of the albumin and globulin preparations from the critical periods of all patients were about the same and both were much higher than the albumin of the improved period. No marked difference in the distribution of all the other amino acids of either the globulin or albumin fractions could be detected.

#### DISCUSSION

It is evident from the results presented here that the dietotherapeutic treatment of nephrosis must take cognizance of the kind and amount of protein lost during the course of the disease. The high proteinuria of the critical period not only involves a greater loss of proteins than the benign period but also a loss of higher quality proteins as indicated by their greater content of essential amino acids. Hence the nutritional stress is qualitatively far greater than the actual quantity of excreted proteins denotes.



TABLE IV COMPARISON OF THE AMINO ACID COMPOSITION OF HUMAN BLOOD PROTEINS, COW'S MILK PROTEINS, AND URINARY PROTEINS OF NEPHROSIS

(All values are given in grams amino acid per 100 Gm. protein)

AMINO ACID	URINARY PROTEINS		HUMAN PLASMA PROTEINS (BRAND <sup>22</sup> )					HUMAN GLOBIN*	CASEIN (AMIGEN*)	LACTALBUMIN (EDAMIN*)
	ALBUMIN	GLOBULIN	ALBUMIN	$\gamma$ GLOBULIN	$\beta$ GLOBULIN	$\alpha$ GLOBULIN	FIBRINOGEN			
Arginine	5.4	5.5	6.2	4.8	6.8	7.7	7.9	3.3	2.4	3.2
Histidine	3.4	3.1	3.5	2.5	2.8	2.8	2.8	3.5	3.0	3.1
Methionine	2.8	3.5	1.3	1.1	1.7	1.4	2.5	2.5	2.5	2.6
Cystine	5.4	3.7	5.6	2.4	3.4	1.5	2.3	0.3	0.4	1.2
Phenylalanine	5.9	7.1	7.8	4.6	4.7	4.6	4.2	6.1	4.8	3.7
Tyrosine	4.5	4.0	4.7	6.8	6.0	4.5	5.8	2.7	0.9	3.7
Isoleucine	3.3	5.3	1.7	2.7	5.0	1.7	4.8	1.0	4.6	4.1
Tryptophane	1.8	2.7	(0.2)	2.9	2.0	1.9	3.3	1.2	2.4	2.3

\*These amino acid values were obtained in this laboratory by the use of the same methods employed for the analyses of the urinary proteins

On the basis of our evidence, it seems that some suggestions can be made as to the kind of proteins or amino acid mixtures which might be expected to alleviate better the amino acid depletion induced by the disease. Thus, if we compile the average composition of urinary proteins collected during the critical phases of the disease (albumin globulin ratios 1.68 to 3.30) and compare these amino acid values with those of proteins which have been employed in the treatment of nephrosis, a measure of their possible efficacy can be gained (Table IV). It is clear from these data that none of the human blood proteins provide amino acids in the general pattern in which they occur in the urinary proteins<sup>4, 5, 6</sup>. Attention is called to the methionine, isoleucine, and tryptophane deficiencies of plasma albumin, the methionine, cystine, phenylalanine, and isoleucine deficiencies of the globulins, and the cystine and phenylalanine deficiencies of fibrinogen. Human globin is poor in cystine and isoleucine by comparison with urinary albumin and globulin. Turning to the milk proteins we find that casein (Amigen) is lacking in cystine, phenylalanine, and tyrosine,<sup>3</sup> whereas lactalbumin (Edamin) is low only in cystine and phenylalanine. Of course any protein given in large enough quantities will compensate for the urinary protein losses, but this practice involves the administration of large quantities of unnecessary amino acids which the already metabolically embarrassed organism must degrade and thereby inevitably augment the prevailing high blood nonprotein nitrogen beyond the limits of tolerance. In view of these considerations it would seem more reasonable to assay the value of available digests which have been supplemented with pure amino acids to simulate the amino acid configuration of the urinary proteins.

In addition to this practical application, these findings also serve to answer in part the question of the origin of the proteins excreted in nephrosis. A comparison of the amino acid composition of the urinary proteins and the normal blood proteins (Table IV) indicates that neither the urinary albumin or globulin of the critical period could arise respectively from the plasma albumin or any mixture of the plasma globulins. This leads to the surmise

that the hypoproteinemia of nephrosis does not result primarily from a loss of blood proteins into the urine but from the relegation of amino acids to the formation of the aberrant proteins which are excreted into the urine and abdominal cavity

#### SUMMARY

Amino acid analyses of the proteins found in the urine and abdominal cavity of patients suffering from nephrosis reveal that the urinary albumin and globulin have a different amino acid pattern from the plasma albumin and globulin. This finding suggests that the hypoproteinemia of the disease is due to the utilization of amino acids for the formation of the aberrant proteins occurring in the excretory fluids rather than the excretion of normal blood proteins. The further suggestion is made that protein preparations which simulate the amino acid patterns of the urinary proteins be assayed in the dietotherapy of the disease.

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## NUTRITIONAL STATUS AND INFECTION RESPONSE

### II ELECTROPHORETIC CIRCULATING PLASMA PROTEIN, HEMATOLOGIC, HEMATO- CHEMISTIC, AND PATHOLOGIC RESPONSES TO MYCOBACTERIUM TUBERCULOSIS (H37RV) INFECTION IN THE PROTEIN DEFICIENT RAT

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#### INTRODUCTION

THE concept that malnutrition increases susceptibility or reduces resistance to tuberculosis gained impetus after the World War of 1914 to 1918. Increased mortality in Europe to tuberculosis during that period was ascribed by epidemiologic inference largely to nutritional deficiencies—particularly deprivation of proteins and fats.<sup>1-4</sup> These epidemiologic observations have not been supported by controlled laboratory experiments. An adequate experimental approach however is difficult to achieve for chronic tuberculosis occurring in man is somewhat different from the disease produced in experimental animals.

Since a multiplicity of factors undoubtedly influence the susceptibility, resistance, and course of tuberculosis it seemed desirable to approach experimentally the problem of dietary deficiency in relation to resistance to and course of this disease by using an ordinarily resistant animal species. The albino rat was chosen since its diet could be well evaluated. It has a relatively high resistance to experimental tuberculosis. When appropriately infected by virulent strains of tubercle bacilli granulomatous lesions occur although rats rarely die of the disease. Employment of the rat in the study of problems associated with tuberculosis is not unique.<sup>5-8</sup> Several of these studies were concerned with the influence of nutritional factors upon experimental tuberculosis.<sup>10-14</sup> only two with the effect of protein deficiency.<sup>15, 16</sup> Because of the current interest regarding the relation of protein deficiency to infection response stimulated by Cannon<sup>17, 18</sup> it seemed desirable to review this particular phase of the tuberculosis problem utilizing more recently available techniques. Accordingly controlled studies were done embracing several concomitant phases of physiologic and pathologic responses to tuberculosis infection and protein deficiency—including growth food consumption, the concentration total circulating, and electrophoretic distribution of plasma proteins hematologic and hematopoietic variations, and histologic tissue response. Well nourished and chronically protein deficient rats, as well as animals with subsequently superimposed acute severe protein deprivation were studied.

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## METHODS

In preliminary experiments, consistent infection was obtained and physiologic and pathologic responses were noted. In this experiment, sixty-four young, growing, male, Sherman strain rats (60 to 70 grams) were used. These were isolated in individual wire mesh cages and kept in a room maintained at 75° F. After an initial seven-day acclimatization period on a basal 18 per cent casein diet,\* the animals were divided into two major diet groups. Thirty-one rats were continued on the basal 18 per cent casein diet. Thirty-three rats were reduced to an 8 per cent casein diet.\* After two weeks on these control and moderately protein deficient diets, the animals were divided into experimental groups and inoculated as follows:

DIET	INFECTED	GROUP	
		Heat killed organisms	NONINFECTED Diet control no organisms
18% protein level	19	6	6
8% protein level	20	6	7

Infection was accomplished by direct intrajugular injection of 0.05 cc of a suspension containing 0.1 mg of a young culture of virulent, viable, human strain (H37RV) bacilli.† A similar suspension of heat-killed organisms was injected intrajugularly as an infection and tissue response control procedure. A third group of animals served solely as diet controls. Four weeks (twenty-eight days) after infection and six weeks after the experimental diets were begun, approximately half the remaining infected and control animals of each diet group were reduced acutely to a 2 per cent casein level. Blood samples for the various studies were drawn from an appropriate sample of the respective diet groups prior to infection and before groups of animals were sacrificed at fourteen, twenty-eight, forty-two, and fifty-eight days after infection. One milligram of old tuberculin (O.T.) was injected intracutaneously in all rats forty-eight hours prior to sacrifice.

\*The experimental diets were isocaloric and similar to those used in previously reported studies.<sup>42</sup> They had the following composition:

DIET COMPOSITION PER 100 GM. RATION

FOOD	ADEQUATE RATION (18% CASEIN)			MODERATELY DEFICIENT RATION (8% CASEIN)			DEFICIENT RATION (2% CASEIN)		
	18 Gm	8 Gm	2 Gm	18 Gm	8 Gm	2 Gm	18 Gm	8 Gm	2 Gm
Casein	18 Gm	8 Gm	2 Gm	18 Gm	8 Gm	2 Gm	18 Gm	8 Gm	2 Gm
Sucrose	73 Gm	83 Gm	89 Gm	73 Gm	83 Gm	89 Gm	73 Gm	83 Gm	89 Gm
Mazola	5 Gm	5 Gm	5 Gm	5 Gm	5 Gm	5 Gm	5 Gm	5 Gm	5 Gm
Phillips & Harts salts	4 Gm	4 Gm	4 Gm	4 Gm	4 Gm	4 Gm	4 Gm	4 Gm	4 Gm
Cystine	0.2 Gm	0.2 Gm	0.2 Gm	0.2 Gm	0.2 Gm	0.2 Gm	0.2 Gm	0.2 Gm	0.2 Gm
Thiamine Cl	200 γ	200 γ	400 γ	200 γ	200 γ	400 γ	200 γ	200 γ	400 γ
Pyridoxine	200 γ	200 γ	400 γ	200 γ	200 γ	400 γ	200 γ	200 γ	400 γ
Riboflavin	400 γ	400 γ	800 γ	400 γ	400 γ	800 γ	400 γ	400 γ	800 γ
Niacin	25 mg	25 mg	50 mg	25 mg	25 mg	50 mg	25 mg	25 mg	50 mg
Ca pantothenate	15 mg	15 mg	30 mg	15 mg	15 mg	30 mg	15 mg	15 mg	30 mg
Choline Cl	100 mg	100 mg	200 mg	100 mg	100 mg	200 mg	100 mg	100 mg	200 mg
Inositol	100 mg	100 mg	200 mg	100 mg	100 mg	200 mg	100 mg	100 mg	200 mg
Biotin	50 γ	50 γ	100 γ	50 γ	50 γ	100 γ	50 γ	50 γ	100 γ

Halker oil was fed in 0.1 cc amounts biweekly. Water was offered ad libitum. Diets provided 4.1 calories per gram ration. The 18 per cent casein diet afforded 134 to 431 mg N per twenty-four hours per 100 gram rat; the 8 per cent diet 68 to 177 mg; the 2 per cent diet 15 to 34 milligrams.

†The subculture used was derived from the original strain isolated in 1904.<sup>42</sup> The viability of the subculture was satisfactorily demonstrated by inoculation of five guinea pigs with a thirteen-day subculture grown in a liquid medium.<sup>42</sup> After several animal passages, a Dubos media young (seven-day) growth representing a fifth subculture was removed from the media by centrifugation, washed three times with Ringer's solution, and weighed. The organisms were resuspended in Ringer's solution. An even suspension containing 0.1 mg per 0.05 cc was used for injection. A similar heat-killed suspension was prepared by autoclaving. The heat-killed organisms retained morphologic characteristics. In the preliminary experiments, bacilli were recovered from infected tissue cultured and subsequently inoculated into guinea pigs producing the typical disease pattern in these susceptible animals.

Hemoglobin (oxyhemoglobin) was determined with the Klett Summerson photoelectric colorimeter (using a 540 m $\mu$  filter). The relative cell volume was calculated from it.<sup>48</sup> Total leucocyte and differential counts were done by the usual methods. Total plasma protein concentration was determined gravimetrically by the copper sulfate method.<sup>49</sup> Blood volume partition studies, using Evans blue dye T 1824 and a modified single sample photoelectric microcolorimetric technique<sup>48</sup> were done concurrently with the above mentioned concentration determinations. Unit circulating proteins and hemoglobin values were readily calculated by adjusting total volumes to a unit surface area (milliliters per 100 cm SA).<sup>50</sup>

Sera from the sacrificed animal were pooled by groups for electrophoretic analyses. Electrophoreses were carried out over a 1.0 minute period in a modified Tiselius apparatus, according to the technique of the Department of Physical Chemistry.<sup>51</sup> Owing to poor light transmission of the dyed plasma a 1.0 per cent protein concentration was found more practical than previously used 2 per cent concentrations. No significant variation in pattern composition would be expected to result from this more dilute protein concentration. The 1.5 per cent solution was obtained by dilution with and dialysis against sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength. Mobilities were calculated from the descending boundary patterns of five times enlarged schlieren diagrams. Compositional data represent an average derived from planimetric resolution of both ascending and descending boundary patterns excluding the delta and epsilon boundary anomalies.

Bone marrow imprints<sup>52</sup> were obtained from the distal diaphyseal third of the left femur when the animals were sacrificed. Eight to ten consecutive imprints were made on each of three cover slips. In addition to the usual May-Grunwald-Giesma stains, occasional sections were stained with the Ziehl-Neelsen technique. Cytologic classification and grouping were as previously described.<sup>4</sup>

At specified intervals infected and control animals were killed by ether anesthesia and all viscera grossly examined. The tissues were then fixed in Zenker's fixative for twenty-four hours and washed in tap water for a similar period, and paraffin blocks were prepared. Routine sections were made of lungs, tracheobronchial nodes, heart, liver, spleen, kidneys, adrenals, testes, and skin at the site of inoculation with O.T. The intestine, pancreas, and mesenteric nodes of a few animals were sectioned at random. All sections were stained with eosin-methylene blue and where indicated, duplicate sections were stained by the Ziehl-Neelsen technique for tubercle bacilli in tissues.

## RESULTS

### I Pathology—

#### a Gross Examination

At autopsy no significant gross changes were noted in the noninfected rats, regardless of dietary status. Several rats died spontaneously of intercurrent infection or trauma incident to cardiac puncture. The latter procedure frequently accounted for focal pulmonary hemorrhage. An occasional intercurrent infection of the lungs obscured gross lesions commonly evident in experimentally infected animals,<sup>54</sup> but did not interfere with the demonstration of *Mycobacterium tuberculosis* in the tissues. Specific gross lesions were consistently demonstrable in lungs and spleen of infected animals. In several control rats, white specks (microscopically identified as lymphoid tissue) dotted the pleural surfaces of the lungs, these nodules usually were not greater than 0.5 mm in diameter. No appreciable pathologic differences existed between control and infected animals at two weeks. However as the postinfection period lengthened smooth, slightly elevated gray-white opaque nodules became conspicuous and progressively larger on the pleural surfaces of the lungs of infected rats. These nodules attained an average diameter of 2 mm eight weeks after infection.

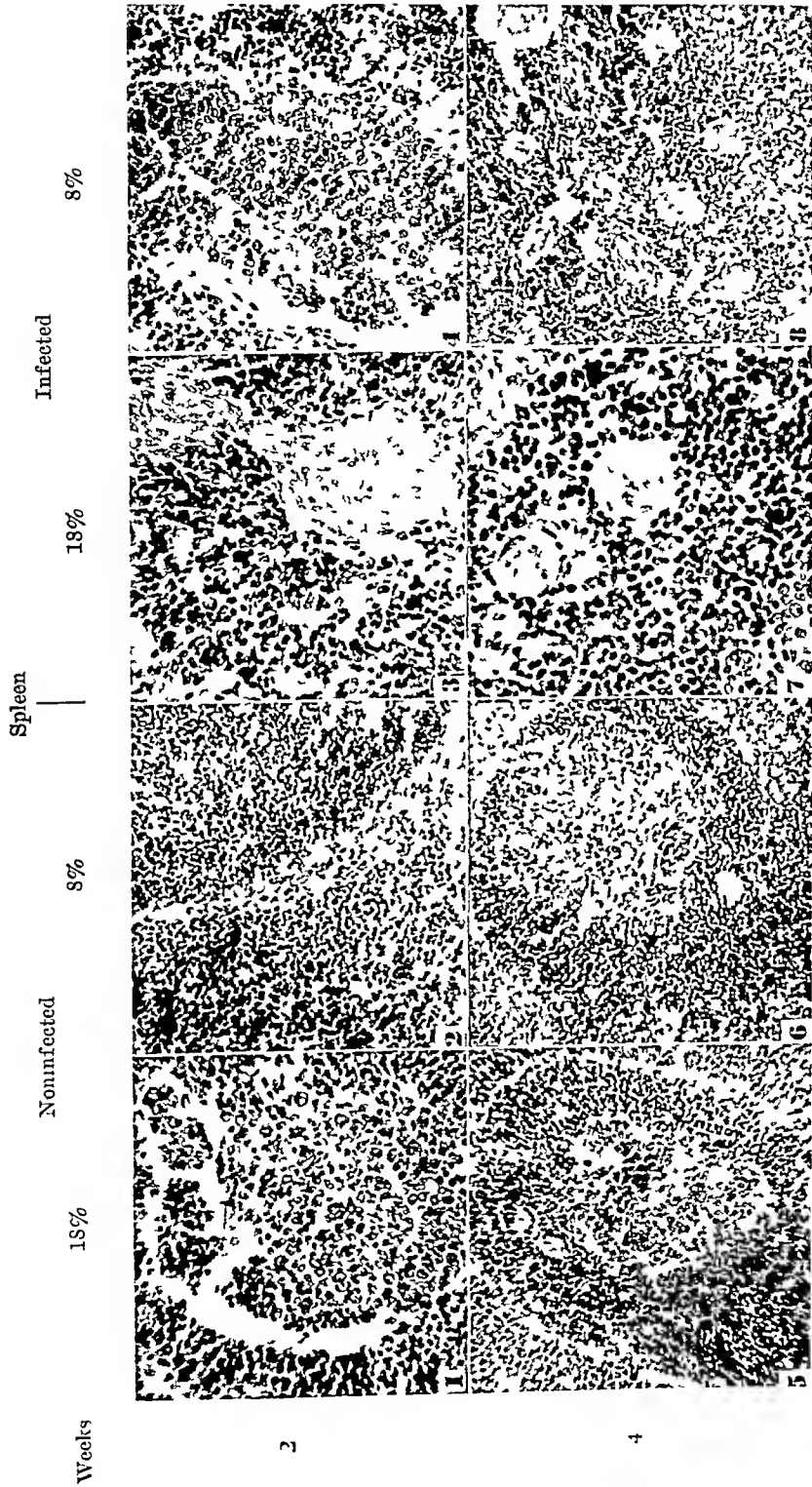




FIG. 1A

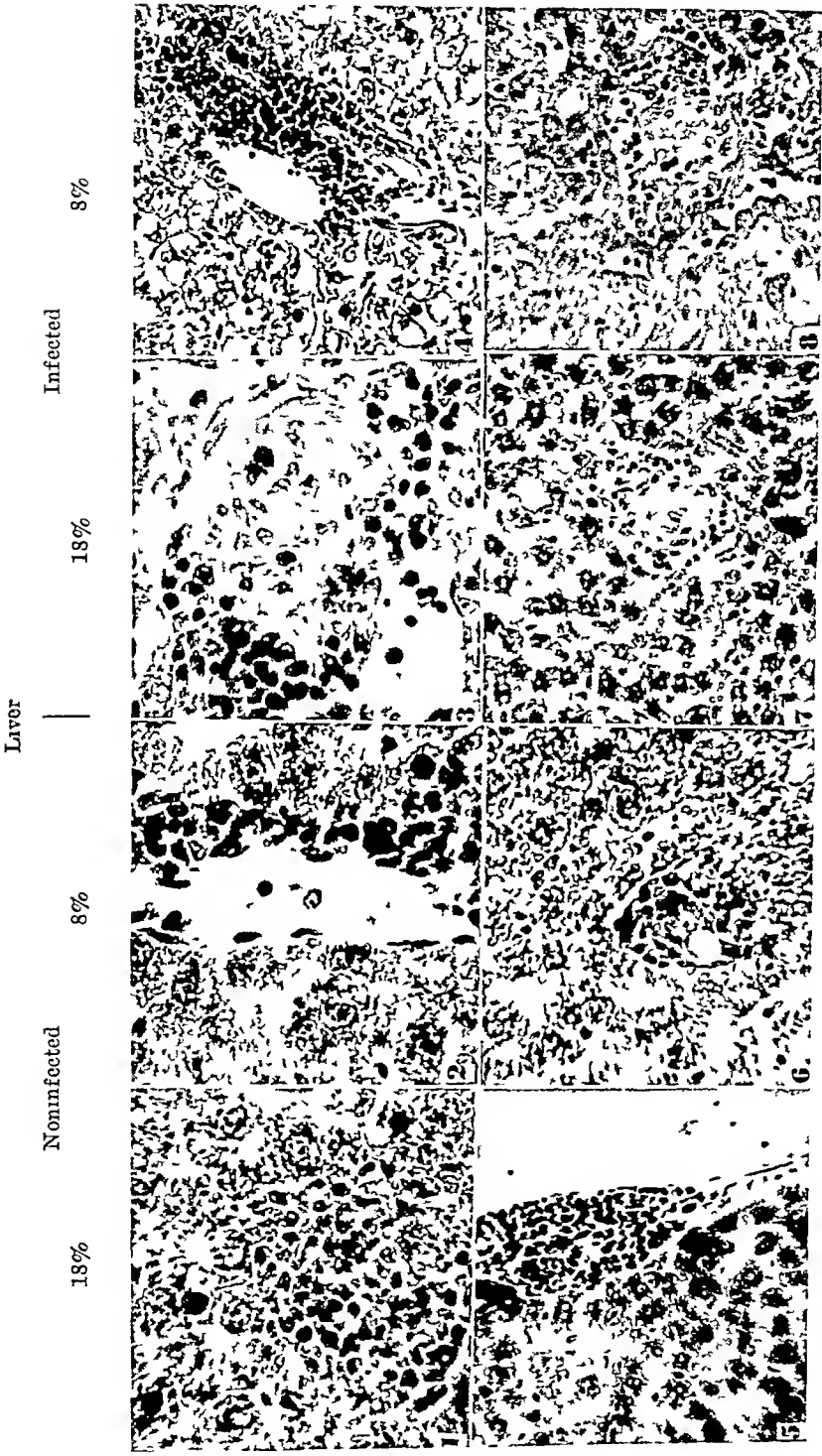
Note. 18% and 18% refer to diet protein levels. weeks refers to weeks postinfection. 8.8 refers to animals reduced acutely to % diet protein levels two weeks prior to sacrifice at six to eight weeks after infection.

#### Spleen

1 (X300) Large germinal center in Malpighian follicle nodules in splenic pulp. No definite marginal zone lymphocyte infiltration. 2 (X60) Follicle architecture distorted by presence of numerous phagocytes. Few lymphocytes at periphery. 3 (X300) Large germinal center in Malpighian follicle. 4 (X170) Large germinal center in Malpighian follicle. 5 (X180) Normal node. 6 (X170) Large germinal center in Malpighian follicle. 7 (X300) Small node adjacent to central arteriole. 8 (X155) Numerous intracellular nodules. 9 (X510) Incubated with heat killed bacilli. Dense histiocytic infiltration both within and surrounding splenic nodules. 10 (X510) Incubated with heat killed bacilli. Dense histiocytic infiltration both within and surrounding splenic nodules. 11 (X70) Small node in periphery. 12 (X70) Small node in periphery. 13 (X170) Incubated with heat killed bacilli. Dense histiocytic infiltration both within and surrounding splenic nodules. 14 (X170) Incubated with heat killed bacilli. Dense histiocytic infiltration both within and surrounding splenic nodules. 15 (X170) Incubated with heat killed bacilli. Dense histiocytic infiltration both within and surrounding splenic nodules. 16 (X700) Extra Malpighian nodule. No central breakdown. Very little cellular reaction.

**Summary of Spleen Pathology** Nodules in the spleen produced by *Mycobacterium tuberculosis* were histologically similar to the seen in other organs and were more numerous within the Malpighian follicles. So little exudative cellular reaction was seen in association with these nodules in the spleen that they frequently appeared as foreign bodies completely surrounded by normal splenic tissue. Lesions in the spleens of animals fed diets deficient in protein were not significantly different from those lesions seen in well fed animals. A reduction of lymphoid tissue in general however no constant changes due to protein deficiency were reflected histologically in the spleen.

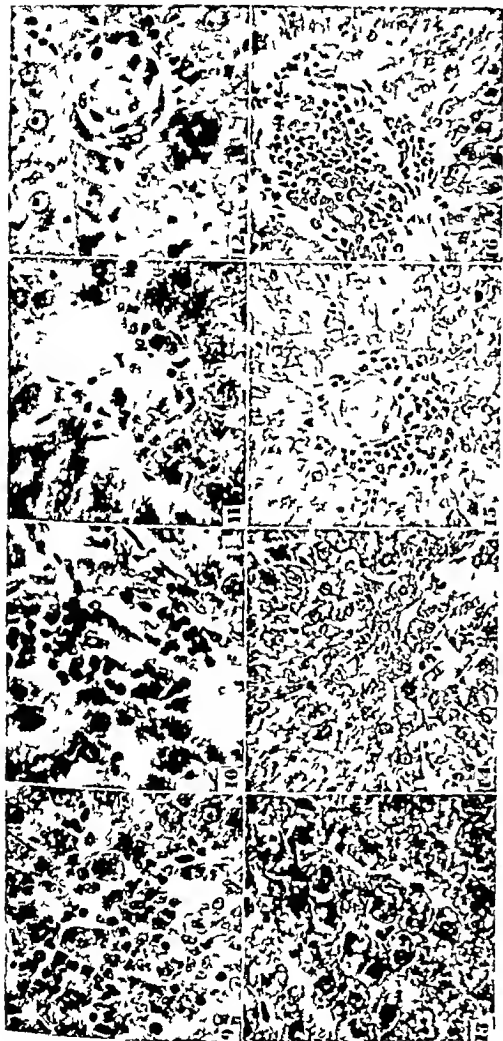




Liver

Fig 1B

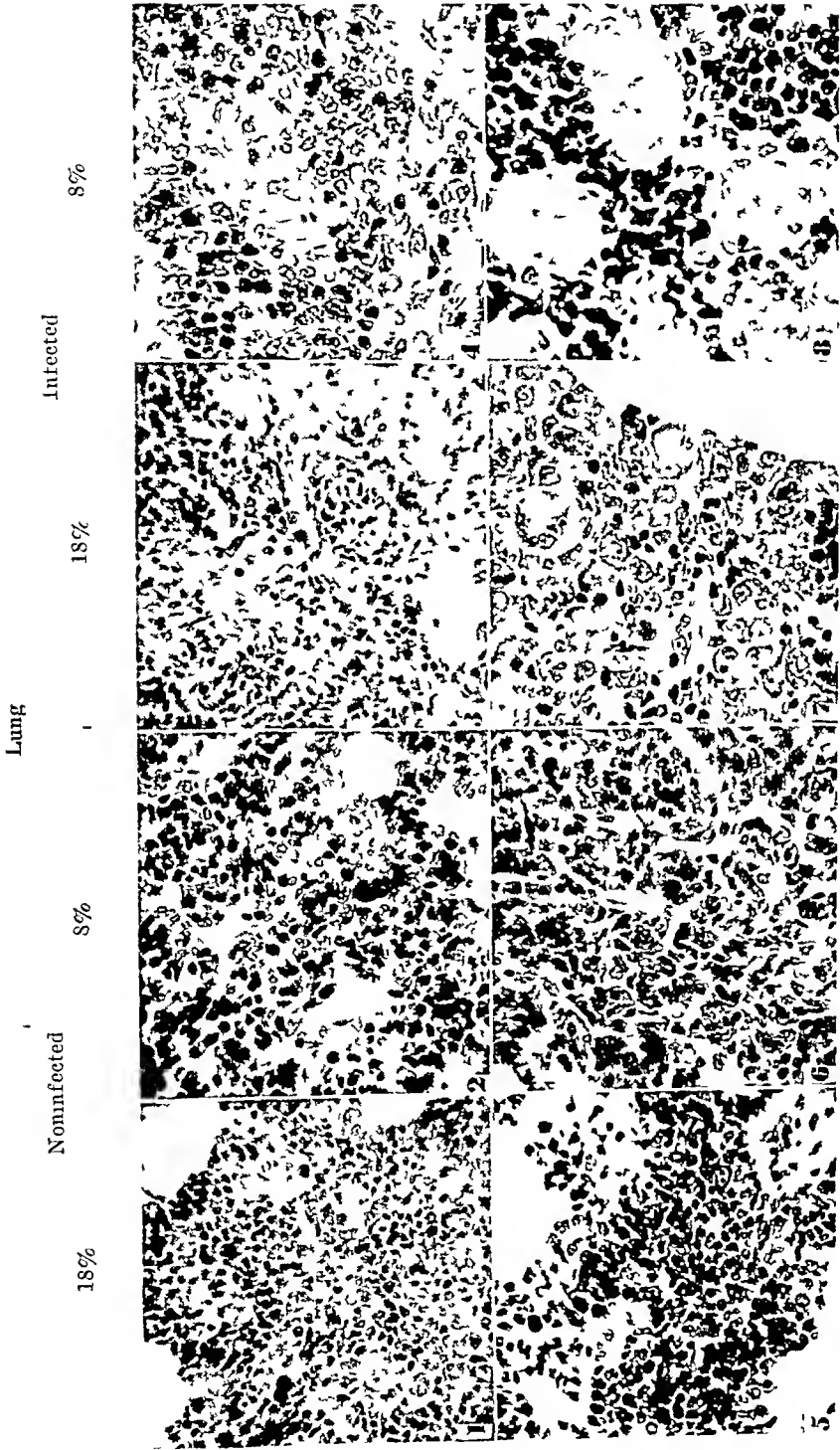
1 (X530) Diet infection control. Focus of round cell infiltration lymphocytes and Kupffer cell derivatives. No nodule formation.  
2 (X720) Round cell infiltration at lobule periphery. early hyperchromatism of liver cell nuclei and swelling of cytoplasm due to protein deficiency 3 (X680) Nodule with surrounding zone of lymphocytes and Kupfferian derivatives. Liver cells well nourished. Sinusoids widely patent 4 (X235) Perivascular lymphoid tissue. No evidence of tuberculous localization. Liver cells show marked protein depletion



5 ( $\times 237$ ) Similar to 1. Adjacent to portal vein no evidence of protein deficiency 6 ( $\times 470$ ) More advanced protein deficiency showing cellular rarefaction and obliteration of sinusoid 7 ( $\times 40$ ) Similar to 3 with less cellular reaction. No evidence of protein deficiency 8 ( $\times 40$ ) Similar to 3 with less cellular reaction. No evidence of protein deficiency 9 ( $\times 210$ ) This diet infection control was inoculated with heat killed bacilli. Sinusoidal cellular infiltration near central vein. No evidence of granuloma. 10 ( $\times 880$ ) Diet infection control inoculated with heat killed bacilli. Sinusoidal cellular infiltration near central vein. No evidence of granuloma. 11 ( $\times 530$ ) Everted liver lesion showing consequences of epithelial cellular infiltration. No histologic evidence of protein deficiency. 12 ( $\times 700$ ) Well developed nodule in sinusoid. No surrounding cellular infiltration. Sinusoidal rarefaction. Liver cells show no dietary inoculation. 13 ( $\times 430$ ) Inoculated with heat killed bacilli. Hyperchromatic nuclei, double nuclei and cytoplasmic rarefaction. Sinusoidal obliteration. Liver cells show no dietary inoculation. 14 ( $\times 330$ ) Dense circumscribed nodule with slight cellular infiltration. In spite of advanced protein deficiency, evidence of no granuloma in this lesion over those in photographs 3 or 11 can be demonstrated. 15 ( $\times 310$ ) Similar to photograph 4 except that liver cells show marked protein deficiency.

**Summary of Liver Pathology** Nonspecific lesions are encountered in rats from time to time as in other laboratory animals and some of the are shown in the livers of control animal. In animals infected with *Mycobacterium tuberculosis* lesions appear early (photograph 3) and are usually well localized when first seen. No appreciable changes in character of liver lesions can be demonstrated after the second week of infection even in animals which have been maintained on diets not richly or fully adequate in protein. The nonspecific lesions are rather constant in the majority of cytoplasm of liver cells and the changes are rather constant. These consisted of swelling and rarefaction of cytoplasm of liver cells, peripheral displacement and gradual appearance of ribonucleic acid granules in the less acutely deficient animals. These changes were present but not so constant in relatively deficient or extremely deficient in protein.

As in the lung, numerous active and latent foci of lymphoid tissue are thought to exist in the livers of normal well fed rats.



Lung

Fig 1C

1 (X310) Nonspecific alveolitis. Many alveolar phagocytes but no nodule formation. A frequent finding in control animals. 2, (X420) Atelectatic lung. Few histiocytes but no specific pattern. 3 (X240) Focus of intra-alveolar nodule formation resulting from coalescence of histiocytes many of which are shown in photograph. 4 (X590) Nodule with early central necrosis but no true caseation



In infected animals rounding of the normally sharp borders of the spleen resulted from diffuse enlargement to approximately twice normal size at the end of eight weeks of infection. Tiny, poorly defined, pinhead sized nodules projected slightly above the surface, and the entire organ assumed an opaque translucency resembling glazed orange rind.

Varying degrees of involution of the thymus were noted in all animals after pubescence. The skin of the abdomen at the site of tuberculin inoculation showed slight induration and a dry, brown crusting in many of the control as well as infected animals after forty eight hours. No orythoma was noted at the periphery of the indurated area in any animal. Systemic reactions due to injection of tuberculin<sup>26, 31, 38</sup> were not observed.

#### *b Microscopic Examination—Noninfected Animals*

A study of the histology of the organs of thirty young control rats revealed several anatomical characteristics which merit brief comment.

**Lymphoid Tissue** Lymphoid tissue was widely distributed throughout the lung parenchyma where it assumed the form of peribronchial and perivascular "collars." Occasionally, in these zones, well developed germinal centers formed large nodules. The tracheobronchial nodes were well developed, in some instances with prominent primary and secondary follicles. The lymphoid tissue of the spleen was characteristically dense, particularly the Malpighian follicles in which large, clearly demarcated secondary follicles were consistently present. In other organs, such as the liver, lymphoid tissue was less conspicuous, usually consisting of aggregates of lymphocytes and macrophages adjacent to the trabeculae.

**Lungs** The lungs of all rats commonly revealed large cells having small, round or oval vesicular nuclei and extremely pale cytoplasm. Frequently, the cytoplasm was identified only by the demarcating cell membrane, occasionally it appeared unusually foamy. These cells were either present in alveolar septa or lying free in alveolar air spaces. In the septa, they appeared closely associated with lymphoid tissue. In the air spaces, they rather closely resembled the alveolar phagocytes, or heart failure cells, often seen in human lungs. These cells are thought to play an important role in the reaction of the rat to infection with *Mycobacterium tuberculosis*.<sup>39</sup>

**Liver** An inconstant, though frequent, degenerative change was noted in the livers of rats subjected to severe dietary protein deficiency. Hepatocellular degeneration was evident in the peripheral areas of the lobules. It was characterized by liver cell swelling, cytoplasmic rarefaction, frequent hyperchromatism of nuclei, and a conspicuous cell membrane. Because of the cellular swelling, the sinusoids in these areas seemed obliterated. Basophilic granules (presumably representing ribonucleic acid<sup>55, 56, 57</sup>) were displaced peripherally in the cytoplasm of less severely involved cells and had disappeared entirely in cells showing advanced swelling and rarefaction. Similar hepatic cell changes have been noted in both dogs and rats as a result of protein deficient diets.<sup>59, 60</sup> In this laboratory, such lesions have subsequently been produced within three days in rats on a protein free diet.<sup>61</sup>

#### *c Microscopic Examination—Infected Animals*

**Lymphoid Tissue** In contrast to the findings of Lange and Simmonds,<sup>19</sup> lesions in the tracheobronchial lymph nodes usually seemed to be uniformly distributed throughout the cortical and medullary portions and consisted of discrete nodules made up of eight to twelve epithelioid cells which had coalesced. As in the lungs, no progression of lesions beyond this stage was observed. Lesions similar to those of the tracheobronchial nodes were found in the spleen. These principally occurred in germinal centers of Malpighian follicles, although frequently epithelioid cell nodules were seen in the red pulp. Lymphocytes were relatively rare adjacent to the lesions. Neither necrosis nor coalescence of nodules was observed.

**Lungs** The smallest tuberculous lesion consisted of a group of three or four epithelioid cells which had coalesced to form a tiny nodule. These epithelioid cells seemed identical with those described as alveolar phagocytes in many of the control animals (Fig. 1). The coalesced cell nodules usually could not be characterized as typical Langhans' giant cells since the nuclei remained well defined and central. Occasional typical Langhans'

cells were noted. In many instances lesions were regularly disposed beneath the pleura at intervals of 2 to 3 mm, frequently in or near peribronchial or perivascular lymphoid tissue. Most lesions showed a few lymphocytes and macrophages at their margins. Occasionally a ring of lymphocytes surrounded the epithelioid cell nodule. In most instances this arrangement could be seen to result from the multicentric development in lymphatic\* or lymphoid tissue of epithelioid cell nodules which compressed the intervening lymphocytes.

Progression of the lesion from this stage consisted merely of coalescence of the syncytial masses into larger nodules constituting a nodule (Fig 1). An occasional epithelioid cell contained a rather pale nucleus and more eosinophilic cytoplasm than was evident in neighboring cell, but frank necrosis was not noted in any lesion. No further tendency toward regression or localization of lesions (e.g. walled off or circumscribed by fibroblastic proliferation) was noted after eight weeks of infection than was evident one week after infection.

**Liver** In the liver similar but relatively fewer lesions than were found in the lungs, tracheobronchial nodes, or spleen were noted. Characteristically the lesions were in or very near the trabeculae and usually consisted of a single epithelioid cell nodule of six to ten cells surrounded by a narrow zone of lymphocytes and a few macrophages. In one rat, larger lesions composed of coalescent nodules were noted (Fig 1).

**Kidneys** Kidney lesions were encountered in two rats. One occurred in the cortex and the other in a renal papilla. Both lesions were similar to those usually found in the lungs and liver and consisted of several coalescent epithelioid cell nodules, each surrounded by a narrow zone of lymphocytes and macrophages.

**Testes** The single lesion found in one testicle was similar to those noted elsewhere.

No lesions were found in the intestine or adrenals. Exudates were present only in the serous cavities of two animals which died spontaneously of intercurrent infection.

**Skin** All animals irrespective of infection or dietary status showed considerable reaction to O.T. injected intracutaneously. Dense round cell infiltration consisting largely of macrophages and lymphocytes occurred in the deeper layers of the dermis and subcutaneous tissues in most rats. Edema and slight hemorrhage were commonly seen in the subcutaneous tissues at the site of injection. Local ulceration of the skin was more consistently present in infected and chronically protein deficient animals than in any other group. A few neutrophils invariably were seen near the base of such ulcerations.

**Organisms** In most lesions tubercle bacilli were found in large numbers (Table I) and could often be seen under low power (Fig 1). Most commonly, organisms were found

TABLE I ESTIMATED AVERAGE INCIDENCE\* OF TUBERCLE BACILLI IN TISSUES OF INFECTED RATS

WEEKS AFTER INFECTION	NUMBER OF ANIMALS PER GROUP		LUNG		PERIBRONCHIAL NODES		LIVER		SPLEEN	
	DEFI CIENT†	CON TROL†								
2	0b	0	++b	+++	++b	+++a	++b	++d	++b	+++
4	0	0	+++	++	+++a	+++	+++a	++d	+++	+++a
6-8	4	3	++d	+++a	++b	+++a	0	0	++b	++b
6-8†	4	6	++d	++b	++a	+	0	0	++b	++

Graded on basis of 0 to +++

0 = No organisms seen

++ = Organisms found after protracted search

+++ = Organisms found readily but not in great numbers

+++ = Many organisms

†Deficient refers to 8 per cent casein diet control 18 per cent casein

§Both 8 per cent and 18 per cent reduced to 2 per cent casein at four weeks thus representing a superimposed deficiency

a Tissues of one animal in this group revealed no organisms

b Tissues of two animals in this group revealed no organisms

c Tissues of three animals in this group revealed no organisms

d Tissues of only one animal in this group revealed organisms after search

Lymphatic tissue denoted presence of germinal centers

within the vacuolated nodules formed by coalescing epithelioid cells. In the lungs, however, they were also frequently encountered in single mononuclear phagocytic cells situated along the margins of the alveolar air spaces. Intracellular, acid fast debris often was present in epithelioid cell nodules or in single alveolar phagocytes when organisms were not readily found.<sup>31</sup> The average incidence of bacilli in tissues examined histologically

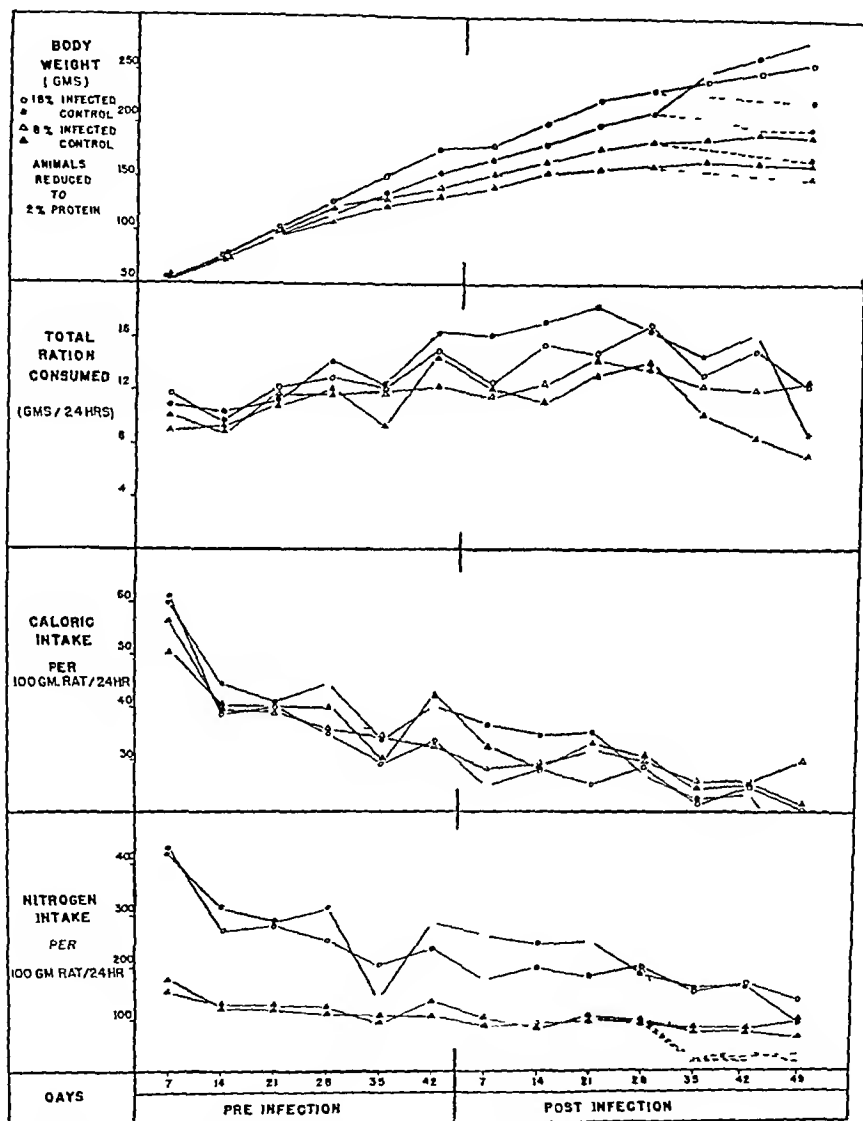


Fig. 2—Alterations in body weight and libitum ration consumption and estimated nitrogen and caloric intakes associated with tuberculous infection and diet protein deficiency as compared with noninfected and diet controls

appeared to decrease rather markedly within six to eight weeks after infection was initiated. Organisms seemed most persistent in peribronchial nodes and spleen. Diet differences exerted no striking influence upon the persistence of organisms in tissues (Table I). Rather rapid disappearance or dissolution of bacilli in the rat has been previously noted<sup>20, 22, 29, 33, 37, 39, 40</sup>. Differences in diet have been thought to influence the persistence of lesions or bacilli by some,<sup>26, 30, 34, 37</sup> not by others.<sup>19, 20, 21, 41</sup>

## II Growth —

**Caloric and Nitrogen Consumption** Weight increments of 3 to 5 grams per day on the initial control diets represent an adequate growth rate for this age, sex, and strain rat on this 16 per cent casein diet. Weight gain increment with increasing age was relatively exponential. Rats subjected to somewhat deficient nitrogen intakes (8 per cent casein diets) continued to grow but at a considerably slower rate than did controls. A weight increment of 15 to 2 grams per day followed initiation of the 8 per cent diet and within nine weeks diminished to a -0.2 to 1.0 gram per day increment. Superimposition of severe protein deficiency resulted in a progressively greater weight loss in the previously well nourished animals. Infection resulted in no apparent disturbance of growth rates irrespective of diet.

Total ration consumed (grams per twenty four hours per rat) was somewhat greater at an 18 per cent than it in 8 per cent diet protein level (12 to 18 versus 9 to 15 gram per twenty four hours). The unit caloric intake (per 100 gram rat per twenty four hours) was similar at both levels of protein intake (21 to 45 calories). The unit caloric intake diminished with increasing age. The estimated nitrogen intake ranged from 150 to 300 mg nitrogen per 100 gram rat per twenty four hours in the well nourished and 75 to 130 mg nitrogen per 100 gram rat per twenty four hours in the moderately deficient rats. Rats with superimposed severe (2 per cent protein) deficiency consumed 15 to 30 mg nitrogen per 100 gram rat per twenty four hours. Infection induced no alteration in ad libitum food consumption. The similarity of unit caloric and nitrogen intakes at both diet protein levels suggests that pair fed nutrition control data are not essential in these experiments. (Fig. 2)

## III Blood Studies —

Normal values for hemoglobin, leucocyte and plasma protein concentrations in rats of this age, sex, and strain have been previously reported.<sup>4, 5</sup> Infection per se did not result in significant alterations in the concentrations of these various components (Table II).

**Total Leucocyte and Differential Counts** Total leucocyte counts did not appear to be remarkably altered as a result of either infection or moderate dietary protein deficiency. The control animal counts in general tended to be slightly higher than reported normal values for other rat strains. They were consistent with values ranging from 10 to 20,000 WBC per cubic millimeter noted in rats of the same age, sex, and strain on identical diets reported in a previous experiment.<sup>4</sup> The range of established 'normal' values for rat differential counts is rather large. The slight intergroup variations noted were well within the established range. The data suggest a tendency toward relative polymorphonuclear leucocytosis with correlative lymphopenia in both well nourished and moderately deficient rats within twenty eight postinfection days. This tendency was neither constant nor persistent. Few monocytes were usually observed in peripheral smears. Variations in these elements were negligible. Accordingly, the theory of monocyte-lymphocyte response as reflected in the peripheral blood in experimental tuberculosis<sup>6, 7, 8</sup> could not be evaluated.



TABLE II AVERAGE CONCENTRATION VARIATIONS OF BLOOD

DAYS OF INFECTION	PREINFECTION PERIOD		14 DAYS				28	
DIET GROUP	18%	8%	18%		8%		18%	
INFECTION STATUS	CONTROL	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL
NUMBER OF ANIMALS	8	8	4	3	5	3	4	3
Hemoglobin <sup>a</sup> (Hb = Gm/100 cc) 12.20 ± 0.9 - 15.1 ± 0.3	15.5	15.3	15.2	15.4	14.1	14.2	15.6	16.7
Total protein <sup>a</sup> (TP = Gm/100 cc) 6.60 ± 0.1 - 8.07 ± 0.07	7.26	7.10	8.22	7.08	6.81	6.87	8.09	7.69
Leucocyte Count <sup>45</sup> (WBC/mm <sup>3</sup> in thousands) 8 - 15,000*	12.1	18.3	22.1	24.0	26.8	26.4	32.2	17.7
Polymorphonuclear leucocytes <sup>45</sup> (%) 4 - 25%	15	12	13	11	15	14	22	15
Lymphocytes <sup>45</sup> (%) 75.95%	82	86	84	95	83	82	78	85

\*Slightly higher total leucocyte counts have been observed in the control rats of this sex and strain.

†Rats previously maintained on 18 per cent casein diet reduced to 2 per cent casein at twenty-eight days.

‡Rats previously maintained on 8 per cent casein diet reduced to two per cent casein at twenty-eight days.

**Hemoglobin** Hemoglobin concentration values for rats of this age, sex, strain, and diet have been established.<sup>50</sup> Observed values showed no significant variation from "normal" irrespective of dietary and/or infection status. Significant changes in available circulating hemoglobin are frequently masked by lack of apparent change in concentration. This phenomenon has been demonstrated previously in relation to protracted moderate protein deficiency.<sup>65</sup> Comparison of initial (preinfection) and final (forty-two to fifty-eight days later) values of unit circulating blood volume and hemoglobin (Table III) suggests that a significant diminution of both attended protein deficiency irrespective

TABLE III AVERAGE VARIATIONS OF CIRCULATING

DAYS OF INFECTION	PREINFECTION PERIOD		POSTINFECTION PERIOD				POSTINFECTION	
DIET GROUP	18%	8%	14 DAYS				28	
INFECTION STATUS	CONTROL	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL
NUMBER OF ANIMALS*	8	8	4	3	5	6	4	3
Surface area (SA = cm <sup>2</sup> )	275.1	241.6	310.0	260.7	278.0	268.2	334.5	278.4
Total blood volume (cc)	11.5	15.3	15.9	10.3	12.6	10.9	13.2	10.1
Unit blood volume (BV <sub>u</sub> = cc/100 cm <sup>2</sup> SA)	4.18	5.28	5.01	3.95	4.49	4.07	3.95	3.63
Unit circulating hemoglobin (CHb <sub>u</sub> = Gm/BV <sub>u</sub> )	0.65	0.82	0.76	0.61	0.63	0.57	0.62	0.60
Total plasma volume (cc)	6.25	6.93	7.29	5.63	7.33	6.34	7.09	5.17
Unit plasma volume (PV <sub>u</sub> = cc/100 cm <sup>2</sup> SA)	2.27	2.70	2.77	2.16	2.62	2.37	2.12	1.84
Unit circulating protein (CP <sub>u</sub> = Gm/PV <sub>u</sub> )	0.18	0.21	0.23	0.15	0.18	0.16	0.17	0.15

\*In general each animal was subjected to one plasma volume study occasionally (twelve times) one rat individuals.

†The number of animals contributing to each mean is small; standard deviations from control means follows: BV<sub>u</sub> = ± 1.07-2.48; BV<sub>u</sub> = ± 0.51-0.57; CHb<sub>u</sub> = ± 0.09-0.10; PV<sub>u</sub> = ± 0.67-1.87; PV<sub>u</sub> = ± 0.38-0.46.

## CELLS AND PLASMA PROTEIN WITH DIET AND INFECTION

DAYS		42-58 DAYS							
8%		18%		18%† 2%		8%		8%† 2%	
INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL
5	2	4	3	5	3	3	3	4	3
101	156	162	167	155	164	153	151	150	144
681	678	769	702	626	611	668	688	638	588
210	216	224	326	301	346	226	265	128	115
10	9	10	10	11	8	11	11	12	21
80	90	89	86	86	89	88	88	88	78

on 18 per cent casein diets  
days  
days

of infection status. No consistent relation between concentration and volume measurements was evident. Experimental tuberculosis per se appeared to exert little significant influence upon unit blood volume or circulating hemoglobin. These findings are consistent with those noted in several patients.<sup>66</sup>

**Hematopoiesis.** The distribution of cellular elements in the examined bone marrows of fifty-seven rats of this series (Table IV) was similar to that noted in fifty-two rats of a previous study<sup>45</sup> and to values reported in twenty-four Wistar strain<sup>3</sup> and twelve Rockland strain<sup>6</sup> rats. The tendency toward an increase in mature myeloid forms with associated peripheral leucopenia pre-

## PROTEIN AND HEMOGLOBIN WITH DIET AND INFECTION\*

PERIOD		POSTINFECTION PERIOD							
DAYS		42-58 DAYS							
8%		18%		18%/2%		8%		8%/2%	
INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	4	3
5	2	4	3	5	3	3	3		
2916	2780	3434	3544	3186	2861	2100	2884	2639	2241
114	121	144	166	115	104	118	111	121	84
380	432	419	469	361	365	440	380	466	382
0	067	068	078	056	060	067	058	069	054
634	656	756	847	629	541	654	613	632	458
17	234	219	239	107	189	242	210	244	209
015	016	016	017	010	013	016	015	015	012

a second was done after a two to three week interval. Data therefore represent determinations on sixty

as previously determined in statistically adequate groups of similar rats under similar conditions were as  
CP = ± 0.02-0.03<sup>45</sup>

TABLE II AVERAGE CONCENTRATION VARIATIONS OF BLOOD

DAYS OF INFECTION	PREINFECTION PERIOD		14 DAYS				28	
DIET GROUP	18%	8%	18%		8%		18%	
INFECTION STATUS	CONTROL	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL
NUMBER OF ANIMALS	8	8	4	3	5	3	4	3
Hemoglobin <sup>50</sup> (Hb = Gm/100 cc) 12 20 ± 0.9 - 15 1 ± 0.3	15.5	15.3	15.2	15.4	14.1	14.2	15.6	16.7
Total protein <sup>50</sup> (TP = Gm/100 cc) 6 60 ± 0.1 - 8 07 ± 0.07	7.26	7.10	8.22	7.08	6.81	6.87	8.09	7.69
Leucocyte Count <sup>45</sup> (WBC/mm <sup>3</sup> in thousand) 8 - 15,000*	12.1	18.3	22.1	24.0	26.8	26.4	32.2	17.7
Polymorphonuclear leucocytes <sup>45</sup> (%) 4 - 25%	15	12	13	11	15	14	22	15
Lymphocytes <sup>45</sup> (%) 75-95%	82	86	84	85	83	82	78	85

\*Slightly higher total leucocyte counts have been observed in the control rats of this sex and strain

†Rats previously maintained on 18 per cent casein diet reduced to 2 per cent casein at twenty eight

‡Rats previously maintained on 8 per cent casein diet reduced to two per cent casein at twenty eight

**Hemoglobin** Hemoglobin concentration values for rats of this age, sex, strain, and diet have been established.<sup>50</sup> Observed values showed no significant variation from "normal" irrespective of dietary and/or infection status. Significant changes in available circulating hemoglobin are frequently masked by lack of apparent change in concentration. This phenomenon has been demonstrated previously in relation to protracted moderate protein deficiency.<sup>65</sup> Comparison of initial (preinfection) and final (forty-two to fifty-eight days later) values of unit circulating blood volume and hemoglobin (Table III) suggests that a significant diminution of both attended protein deficiency irrespective

TABLE III AVERAGE VARIATIONS OF CIRCULATING

DAYS OF INFECTION	PREINFECTION PERIOD		POSTINFECTION PERIOD				POSTINFECTION	
DIET GROUP	18%	8%	14 DAYS		28		28	
INFECTION STATUS	CONTROL	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL	INFECTED	CONTROL
NUMBER OF ANIMALS*	8	8	4	3	5	6	4	3
Surface area (SA = cm <sup>2</sup> )	275.1	241.6	310.0	260.7	278.0	268.2	334.5	278.4
Total blood volume (cc)	11.5	15.3	15.9	10.3	12.6	10.9	13.2	10.1
Unit blood volume (BV <sub>u</sub> = cc/100 cm <sup>2</sup> SA)	4.18	5.28	5.01	3.95	4.49	4.07	3.95	3.63
Unit circulating hemoglobin (CHB <sub>u</sub> = Gm/BV <sub>u</sub> )	0.65	0.82	0.76	0.61	0.63	0.57	0.62	0.60
Total plasma volume (cc)	6.25	6.93	7.29	5.63	7.33	6.34	7.09	5.17
Unit plasma volume (PV <sub>u</sub> = cc/100 cm <sup>2</sup> SA)	2.27	2.70	2.77	2.16	2.62	2.37	2.12	1.84
Unit circulating protein (CP <sub>u</sub> = Gm/PV <sub>u</sub> )	0.18	0.21	0.23	0.15	0.18	0.16	0.17	0.15

\*In general each animal was subjected to one plasma volume study occasionally (twelve times) one rat individuals

†The number of animals contributing to each mean is small standard deviations from control means follows BV<sub>u</sub> = ± 1.07-2.48 BV<sub>u</sub> = ± 0.51-0.57 CHB<sub>u</sub> = ± 0.09-0.10 PV<sub>u</sub> = ± 0.67-1.87 PV<sub>u</sub> = ± 0.38-0.46

A suggestive increase of erythroblasts and/or normoblasts also was manifested early by infected animals. These changes in cellularity were reflected in the myeloid erythroid ratios.

In general, differences in hematopoiesis between the diet and infection groups were not striking, but variations in myeloid erythroid ratios seemed more closely related to infection than to dietary status. However, animals with protracted or severe protein deficiency had slightly lower ratios than well nourished animals, regardless of infective status.

*Plasma Proteins.* Severe protein restriction superimposed upon a previously moderate diet protein deficiency resulted in slight diminution in plasma protein concentration (Table II). Distortion of plasma protein concentration, however, affords a poor indication of variation in circulating proteins, particularly in protein deficiency states. Protein deficiency per se is attended by significant decreases in unit circulating proteins.<sup>6</sup> Severe diet protein deficiency imposed upon a previously well nourished rat appeared to result in greater relative depletion of unit circulating protein than that observed when severe protein restriction was imposed upon a moderately protein deficient rat (Table III). Slight, probably significant relative increases in unit circulating proteins were evident in the first few weeks of the postinfection period in infected rats irrespective of dietary status. Subsequently, similar changes occurred in contrasting groups; hence the relative influence of diet or infection upon the observed circulating protein depletion cannot be assessed or valid inferences drawn. In general, the data suggest that unit circulating proteins were slightly but presumably significantly ( $2 + \sigma$ ) increased in infected animals within the three to four week period following infection.

The electrophoretic distribution of plasma protein components varied with the dietary status. Fairly adequate resolution of the major plasma protein components (albumin, alpha beta plus fibrinogen and gamma globulins) was possible under the conditions of electrophoresis. The precise differentiation of alpha 1 and alpha 2 globulins and of alpha 2 and beta globulins was not always satisfactory in the diluted dye protein solution. Poor demarcation of beta 1 and beta 2 globulins commonly occurred. Beta 2 globulin and fibrinogen were not separated in these analyses. An  $f'$  component<sup>4, 9</sup> with mobility greater than that of albumin was usually apparent. However, assuming six components, gaussian curves were drawn and resolution and proportionate composition of both ascending and descending boundary patterns, excluding the delta and epsilon anomalies, were obtained. In order that the data might be compared with that previously reported in the same strain under rather similar circumstances, extended compositional description was attempted despite the above mentioned limitations. With these reservations, the plasma composition of several experimental groups is presented in Table V. The available data, however, permit comparison of the influence of diet and infection at fourteen and twenty-eight days after infection was initiated and twenty-eight and forty-two days after moderate dietary protein depletion was begun.

TABLE V ELECTROPHORETIC VARIATIONS IN PLASMA

GROUP	NUMBER OF ANI MALS	TOTAL PLASMA PPOTEIN CONCENTRATION (GM %)	PFR CENT PLASMA COMPOSITION*						
			“f”	ALBU MIN	$\alpha_1$	$\alpha$	$\beta_1$	$\beta_2 + \phi$	$\gamma$
18% control <sup>1</sup>	3	6.85	1.2	48.2	14.0	7.4	6.3	17.2	5.6
18% infected <sup>2</sup>	4	7.44	1.8	55.6	11.0	5.8	5.2	15.1	5.5
8% control <sup>1</sup>	3	5.68	2.5	36.6	12.9	12.1	7.1	20.5	8.3
8% infected <sup>2</sup>	5	5.84	2.8	42.8	12.6	9.3	5.8	21.6	5.1
8% infected <sup>1</sup>	5	5.68	1.4	44.4	14.2	8.9	5.5	18.2	7.3

<sup>1</sup> Twenty-eight days after infection initiated forty-two days after protein depletion began in 8 per

<sup>2</sup> Fourteen days after infection initiated twenty-eight days after protein depletion began in 8 per cent

The unit circulating protein composition data in parentheses represent values obtained in another son with the 18 per cent infected rats of this experiment since similar unit circulating protein values were trol group of this experiment remains unexplained

\*Somewhat arbitrary values derived from relative areal resolution and representing average per cent

†Derived from the product of the plasma protein concentration of the pool and the per cent areal com

‡Represents the product of the average unit circulating proteins (Table III) derived from the plasma solved schlieren pattern

§Unit circulating protein (Table III)

As usually noted, protein deficiency was attended by relative decrease in the proportion of plasma albumin. The relative proportions of alpha, beta, and gamma globulins did not appear unusually increased in the well-nourished, infected animals and were similar to observed values in the well-nourished, non-infected group. This observation is in contrast to those reported in tuberculous infection of rabbits<sup>1</sup> and man.<sup>2</sup> However, in the protein-deficient groups, the proportions of alpha-2 and beta globulins seemed increased irrespective of infectious status. Diet deficiency with albumin depletion, commonly associated with the moderately advanced phases of tuberculosis, may account for the reported changes in the globulin fractions, as recently suggested.<sup>3</sup> In accord with this hypothesis, consistent decrease in proportion of albumin in the data of Seibert and co-workers<sup>1, 2</sup> suggests that both animals and patients may have been protein deficient. Constancy of simple serum protein concentration values may be misleading in this regard owing to plasma volume lability and/or alterations in relative proportions of the various components.

Concentrations of the various plasma components in Table V were calculated from the proportionate composition of schlieren patterns and total plasma protein concentrations of the respective pools. Since unit circulating protein data appear to be more reliable than simple concentration measurements as an indicator of plasma protein variation, the total circulating amount of each plasma protein component in the vascular compartment was also calculated and adjusted to unit value. The arbitrary values obtained were dependent upon limitations in techniques of resolution of electrophoretic components, plasma protein concentrations, and mean unit plasma volume determinations. The data, therefore, may be more relative than absolute. The results remain proportional, however, since the errors were largely systematic. Unit circulating plasma protein composition reveals the rather marked albumin depletion attending protein deficiency. With this method of calculation, no significant aberration in any of the unit circulating globulin components was observed in response to tuberculous infection, regardless of diet or degree of albumin depletion.

## PROTEINS WITH TUBERCULOUS INFECTION AND DIET

CONCENTRATION OF PRINCIPAL PLASMA PROTEIN COMPONENTS (gm/100 cc)							UNIT CIRCULATING PLASMA PROTEIN COMPOSITION (cm/unit plasma volume)†							
"f"	ALBU MIN	$\alpha_1$	$\alpha_2$	$\beta_1$	$\beta_2 + \phi$	$\gamma$	f	ALBU MIN	$\alpha$	$\alpha$	$\beta_1$	$\beta + \phi$	$\gamma$	TOTAL (CPA)§
0.08	3.30	0.96	0.51	0.43	1.18	0.38	0.002 (0.006)	0.072 (0.120)	0.021 (0.024)	0.011 (0.012)	0.009 (0.008)	0.026 (0.033)	0.008 (0.016)	0.15 (0.22)
0.13	4.13	0.82	0.43	0.39	1.12	0.41	0.004	0.128	0.025	0.016	0.013	0.035	0.013	0.23
0.14	2.08	0.73	0.69	0.40	1.17	0.47	0.001	0.09	0.021	0.010	0.011	0.033	0.013	0.16
0.16	2.50	0.74	0.54	0.34	1.26	0.30	0.005	0.077	0.023	0.017	0.010	0.039	0.009	0.18
0.08	2.51	0.81	0.51	0.31	1.03	0.41	0.002	0.067	0.021	0.013	0.008	0.027	0.011	0.15

cent diet group

diet group

group of control rats of the same age sex strain and diet<sup>4</sup>. These values are presented for comparison. The unusually low plasma volume and unit circulating proteins of the particular 18 per cent con-

composition of ascending and descending boundary patterns

position of the resolved schlieren pattern

protein concentration plasma volume and surface area and the per cent areal composition of the re-

## COMMENT

The possible relation of tuberculosis to specific nutritional deficiency is attended by considerable practical interest. The chemical, physiologic, immunologic, and clinical attributes of protein have stimulated rather extensive speculation in this regard. Variations in blood or plasma component measurements, particularly plasma proteins associated with this chronic disease would seem to afford information pertaining to effect rather than cause.

The concept of protein deficiency as a primary cause of increased susceptibility or decreased resistance to tuberculosis has arisen by inference largely derived from epidemiologic and favored by clinical experience. The conclusion of Faber is most commonly cited in this regard: "A gross underfeeding with the animal, body building nutritives reduces resistance against tuberculosis."<sup>1</sup> This hypothesis was derived from over all tuberculosis mortality data increments associated with a decrease of from forty two to thirty seven per cent in "animal foodstuff" (protein) composition of a diet affording approximately 2,800 to 3,000 calories during the war years 1914 to 1916. Present concepts of protein nutrition suggest that such a diet be considered reasonably adequate for maintenance of nitrogen equilibrium.<sup>4</sup> The marked variation in host and pathogen response dependent upon species genetic characters, diet factors, and physiologic status in the presence of infection with *Mycobacterium tuberculosis*, not to mention the usual, important, commonly associated socioeconomic considerations, has impeded collection of conclusive experimental data.

In an endeavor to define more closely the possible relation of dietary protein deficiency to inferred alterations in susceptibility to or course of tuberculosis, infection was initiated in the rat under controlled conditions. Several concomitant physiopathologic responses to intravenous inoculation of *Mycobacterium tuberculosis* (H37RV) in rats on adequate dietary protein intakes (nitrogen, 134 to 431 mg N per twenty four hours per 100 gram rat) and moderately deficient protein levels (nitrogen 68 to 177 mg N per twenty four hours per 100 gram rat) were observed and correlated. Since relatively few animals were involved in this particular experiment—thirty nine infected, twelve injected with

heat-killed organisms, and thirteen serving as diet controls—the limited data on biologic material necessitate some degree of statistical inadequacy. Results of this and preliminary experiments manifested reproducible consistency.

Summation of the data would seem to favor the concept that dietary protein deficiency—whether moderate or severe, acute or chronic, superimposed upon a previously adequate or inadequate state of protein nutrition—does not significantly alter the susceptibility, resistance or course of infection with *Mycobacterium tuberculosis* (H37RV) in the young, growing rat under the conditions of this study.

Correlation of these observed phenomena associated with the nutrition and course of experimental tuberculosis suggests that a somewhat similar approach to the problem in the human being may yield useful, objective data. Until such data become available clinical inference derived from observations on nonindigenous disease in the malnourished rat must be attended by considerable reservation.

#### SUMMARY

1 Intravenous inoculation of viable *Mycobacterium tuberculosis* resulted in definite granulomatous lesions in lymphoid tissue, lungs, and liver, characterized by coalesced epithelioid cell nodules, usually with persistent nuclei and ringed by lymphocytes and few macrophages. Necrosis was not observed.

2 The lesions attained maximal development within four weeks after infection. Recognizable organisms had largely disappeared within eight weeks.

3 No significant difference in the form, incidence, or duration of the specific lesion was observed between well-nourished, moderately, or severely protein deficient animals. No deaths attributable to the infection per se were noted in the forty-two or fifty-eight day postinfection period.

4 Changes in total white blood cells, hemoglobin and plasma protein concentrations as well as in circulating hemoglobin and plasma protein, were consistent with those previously observed in protein-deficient animals. No significant variation in the measurements attended infection per se.

5 Bone marrow studies suggested equivocal changes of myeloid-erythroid ratio in infected animals.

6 Electrophoretic analyses of the circulating plasma proteins revealed apparent increases in the alpha and beta globulin components of the simple areal pattern. No such variation was evident when unit circulating quantities of plasma components were estimated. No specific plasma protein changes resulted from infection per se.

7 Under the conditions of this study, dietary protein deficiency did not appear to alter the susceptibility, resistance, course, or physiologic response of the young rat to experimental tuberculosis.

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TABLE V ELECTROPHORETIC VARIATIONS IN PLASMA

GROUP	NUMBER OF ANI MALS	TOTAL PLASMA PROTEIN CONCENTRATION (GM %)	PEP CENT PLASMA COMPOSITION *						
			"f"	ALBU MIN	$\alpha_1$	$\alpha$	$\beta_1$	$\beta_2 + \phi$	$\gamma$
18% control <sup>1</sup>	3	6.85	12	48.2	14.0	7.4	6.3	17.2	5.6
18% infected <sup>2</sup>	4	7.44	18	55.6	11.0	5.8	5.2	15.1	5.5
8% control <sup>1</sup>	3	5.68	25	36.6	12.9	12.1	7.1	20.5	8.3
8% infected <sup>2</sup>	5	5.84	28	42.8	12.6	9.3	5.8	21.6	5.1
8% infected <sup>1</sup>	5	5.68	14	44.4	14.2	8.9	5.5	18.2	7.9

<sup>1</sup> Twenty-eight days after infection initiated forty-two days after protein depletion began in 8 per cent

<sup>2</sup> Fourteen days after infection initiated twenty-eight days after protein depletion began in 8 per cent

The unit circulating protein composition data in parentheses represent values obtained in another group with the 18 per cent infected rats of this experiment since similar unit circulating protein values were obtained in this experiment remains unexplained

\*Somewhat arbitrary values derived from relative areal resolution and representing average per cent

†Derived from the product of the plasma protein concentration of the pool and the per cent areal composition

‡Represents the product of the average unit circulating proteins (Table III) derived from the plasma resolved schlieren pattern

§Unit circulating protein (Table III)

As usually noted, protein deficiency was attended by relative decrease in the proportion of plasma albumin. The relative proportions of alpha, beta, and gamma globulins did not appear unusually increased in the well-nourished, infected animals and were similar to observed values in the well-nourished, non-infected group. This observation is in contrast to those reported in tuberculous infection of rabbits<sup>1</sup> and man.<sup>2</sup> However, in the protein-deficient groups, the proportions of alpha-2 and beta globulins seemed increased irrespective of infectious status. Diet deficiency with albumin depletion, commonly associated with the moderately advanced phases of tuberculosis, may account for the reported changes in the globulin fractions, as recently suggested.<sup>3</sup> In accord with this hypothesis, consistent decrease in proportion of albumin in the data of Seibert and co-workers<sup>1, 2</sup> suggests that both animals and patients may have been protein deficient. Constancy of simple serum protein concentration values may be misleading in this regard owing to plasma volume lability and/or alterations in relative proportions of the various components.

Concentrations of the various plasma components in Table V were calculated from the proportionate composition of schlieren patterns and total plasma protein concentrations of the respective pools. Since unit circulating protein data appear to be more reliable than simple concentration measurements as an indicator of plasma protein variation, the total circulating amount of each plasma protein component in the vascular compartment was also calculated and adjusted to unit value. The arbitrary values obtained were dependent upon limitations in techniques of resolution of electrophoretic components, plasma protein concentrations, and mean unit plasma volume determinations. The data, therefore, may be more relative than absolute. The results remain proportional, however, since the errors were largely systematic. Unit circulating plasma protein composition reveals the rather marked albumin depletion attending protein deficiency. With this method of calculation, no significant aberration in any of the unit circulating globulin components was observed in response to tuberculous infection, regardless of diet or degree of albumin depletion.

TABLE I. STREPTOMYCIN RESISTANCE IN MICROGRAMS PER CUBIC CENTIMETER  
1 GM DAILY DOSE\*

TREATMENT	TUB. STRAIN	STREPTOMYCIN THERAPY (DAYS)										10% STREPTOMYCIN (MG)										DAYS OF TREATMENT
		30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
8	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
14	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110
39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110

\* No growth in 0.5 µg per cubic centimeter of streptomycin.

0.5, 1, 5 etc indicate the last tube in which there was growth in micrograms per cubic centimeter. Growth in 10 µg or more indicates a resistant strain.

tubercle bacilli grown in Dubos liquid media into 5 c c amounts of the same medium containing an increasing number of micrograms of streptomycin per cubic centimeter, i e, 0.5, 1, 2.5, 5, 10, 15, 31, 62, 125, 250, 500, 1,000. A control tube sans streptomycin was added to each test.

The cultures were examined after fourteen days of incubation to determine the concentration of streptomycin in which growth appeared. At this time the control tube showed maximum (four plus) growth.\*

**Blood Levels**—Streptomycin blood levels were determined by the method of Pulaski and Spinz<sup>6</sup>. (A) A six-hour growth of *Klebsiella pneumoniae* in F D A broth (single strength) was diluted 1 to 50 in double strength F D A. One cubic centimeter was then added to 1 c c of serial saline dilutions of the patient's serum to give a total of 2 c c and a final concentration of 1/2 to 1/64 of the serum. (B) As a control, 0.1 c c of a stock solution containing 320  $\mu$ g per cubic centimeter of streptomycin was added to 0.9 c c of pooled normal sera. This was serially diluted with 0.5 c c amounts of sterile saline to give concentrations of 32 to 1  $\mu$ g per cubic centimeter. Five-tenths cubic centimeter of the culture of *K. pneumoniae* diluted 1 to 50 was then added making final dilutions of 16 to 0.5  $\mu$ g per cubic centimeter in a volume of 1 cubic centimeter. (C) After overnight incubation at 37° C the inhibition was determined by noting the last tube (in A and B) in which there was no growth and dividing the control (B) in micrograms per cubic centimeter by the dilution (A) to give the number of micrograms of streptomycin in 1 c c of patient's serum.

## RESULTS

**Resistance in the 1 Gram Group**—Table I shows the highest concentration of streptomycin in which the particular strain of tubercle bacillus grew and the number of days of therapy after which the organisms developed resistance to the antibiotic. Growth in 10  $\mu$ g was the criterion used to indicate streptomycin resistance.

TABLE II SUMMARY OF TABLE I, NUMBER AND PER CENT OF PATIENTS BECOMING RESISTANT AT INTERVALS DURING THERAPY

DAYS	TOTAL PA TIENTS	TOTAL TESTED	TOTAL INDI CATED†	SENSITIVE		RESISTANT		PER CENT RESIS TANT
				TESTED	INDI CATED†	TESTED	INDI CATED†	
Pretherapy	32	32	—	32	—	—	—	0
30	30	13	17	13	17	—	—	0
60	24	15	9	13	8	2	1	13
90	28	17	11	11	9	6	2	29
100-120*	31	25	6	14	3	11	3	45
120	19	19	—	10	—	9	—	48

\*The 120 day total is included in the 100 to 120 day group.

†By indicated is meant the streptomycin resistance which would in all probability result had cultures been obtained and resistances determined i e. in Table I Patient 28 would be considered resistant from ninety days onward for Patient 2 after seventy days there would be no indication for Patient 6 there would be no indication between forty and eighty days and would be 1,000 after ninety days. It should be noted that a few cases have been reported in which cultures have been found to revert from resistant to sensitive.<sup>7,8</sup> However in well over 500 sensitivity titrations from cultures on ninety-six patients we failed to find a single instance of decreased resistance of any significance.

\*A comparison was made between streptomycin resistance readings obtained with the media described and the Tween-albumin medium for submerged-dispersed growth subsequently described by Dubos and Middlebrook.<sup>9</sup> In a total of one hundred duplicate tests there was complete agreement in eighty-eight cases. Of the twelve remaining titrations ten showed higher growth in the newer medium and two in the older. However in only one instance would a strain of tubercle bacillus have been considered sensitive in one media and resistant in the other.

It may be observed from Table I as summarized in Table II, that none of the thirty two strains tested before therapy was resistant to 2.5  $\mu$ g per cubic centimeter of streptomycin. At the end of thirty days of treatment, resistance was still absent in all of thirty patients. However, at the end of sixty days, 13 per cent of twenty four patients had developed resistance; at ninety days 29 per cent of twenty eight patients; and at 100 to 120 days, 45 per cent of thirty one patients had become resistant to streptomycin.

*Resistance in the 1.8 to 2.4 Gm Group*—Previous to the inauguration of the 1 Gm protocol six patients had been treated with 1.8 to 2.4 Gm of streptomycin daily. Tests of these strains after 100 to 120 days of therapy showed that 83 per cent had become resistant to 10  $\mu$ g per cubic centimeter; 17 per cent remaining sensitive.

TABLE III POSTSTREPTOMYCIN RESISTANCE 1.8 TO 2.4 Gm DAILY

PATIENT	DAYS OF TREATMENT	RESISTANCE AT END OF THERAPY ( $\mu$ g/cc)	POSTSTREPTOMYCIN (MO)									
			1	2	3	4	5	6	7	8	9	10
104	120	15					1000	1000	1000			
101	240							1000	1000	1,000		
103	120											1,000
107	120											1
109	220					1000	1000			1000		
110	120									5		
111	120										5	1
112	120					62						31
113	100	10					15	1000	1000			
114	210						1000					
116	120			0.5			0.5	0.5	0.5			
115	120						31	15	15	10		
118	150									0	0	
118A	120	1000		1000								
119	120							0.5		0.5		
132	120			62	62							

118A Second course of treatment for Patient 118

Patients 104, 113, and 118A are cases from Halloran Hospital. All others were transferred to this hospital after completion of streptomycin therapy.

Resistances in micrograms per cubic centimeter. Growth in 10  $\mu$ g per cubic centimeter indicates resistant strain.

A number of patients under the 1.8 to 2.4 Gm treatment (on whom no previous sensitivities had been performed) were transferred to Halloran Hospital after therapy had been completed. Table III indicates the results of post streptomycin resistance tests on thirteen of these transferred patients and three of our own (Patients 104, 113, 118A). It is of interest to note that two patients in this group (104 and 113) as well as two in the 1 Gm group (9 and 16) became considerably more resistant to streptomycin after the completion of therapy.\*

\*These sensitivities were checked and rechecked.

*Serum Levels*—Table IV indicates the range and average level of streptomycin in micrograms per cubic centimeter detected in the serum of the treated patients after thirty days or more of therapy. It may be seen that the average serum level dropped rapidly from 33  $\mu$ g at two hours to 8.6 at eight and then tapered off to 1.7  $\mu$ g at twelve hours.

TABLE IV STREPTOMYCIN SERUM LEVEL IN PATIENTS RECEIVING 1 GRAM (0.5  $\times$  2) DAILY\*

HR AFTER INJECTION	RANGE ( $\mu$ G/CC)	NUMBER OF SPECIMENS	AVERAGE IF VFI
2	16-64	21	33
4	16-32	7	21.3
6	8-16	7	11.5
8	4-16	7	8.6
10	4-8	7	6.0
12	0-4	30	1.7

\*Levels taken after thirty days of therapy.

## DISCUSSION

One of the greatest problems in the streptomycin therapy of tuberculosis is the varying rate of time necessary for the tubercle bacillus to become resistant to this antibiotic. In an effort to combat this difficulty and the effects of toxicity, many variations in the amount of streptomycin, the number of daily injections, and the duration of therapy have been tried, both by the Veterans Administration and other investigators.

TABLE V

INVESTIGATOR	GM PPF DAY	DAYS OF TREATMENT	NUMBER OF CASES	NUMBER SPASITIVE	NUMBER RESISTANT	PER CENT RESISTANT	CONSIDERED RESISTANT IF NOT INHIBITED BY
Muschenheim <sup>7</sup>	3	75-120	17	1	16	94.0	500 $\mu$ g/cc
	1	42	12	10	1	9.0	500 $\mu$ g/cc
McDermott <sup>8</sup>	?	90-100	20	1	19	95.0	500 $\mu$ g/cc
VA <sup>9</sup>	1.8-2	60-120	62	23	39	63.0	20 $\mu$ g/cc
Fisher <sup>4</sup>	1.8	120	20	9	11	55.0	10 $\mu$ g/cc
VA <sup>10</sup>	1.8-2	120	88	19	69	78.0	10 $\mu$ g/cc
		60-90				64.0	10 $\mu$ g/cc
VA <sup>11</sup>	1	90	17			61.0	10 $\mu$ g/cc
		120	17			76.0	10 $\mu$ g/cc
VA <sup>13</sup>	1	61-90	115			43.5	10 $\mu$ g/cc
		91-120	117			62.4	10 $\mu$ g/cc

Table V illustrates some of the results of other investigators. It will be observed that the Veterans Administration<sup>10</sup> obtained 78 per cent resistant strains after 120 days of 1.8 to 2 Gm of streptomycin daily. On the other hand, a regime of 1 Gm for forty-two days<sup>7</sup> is reported to have resulted in 9 per cent resistant organisms at the completion of therapy. Other reports on the 1 Gm dose<sup>11</sup> showed 61 per cent resistant strains at ninety days and 76 per cent at 120 days. The most recent Veterans Administration<sup>13</sup> results indicate 43.5 per cent of 115 patients resistant sixty-one to ninety days after the beginning of treatment with 1 Gm daily.

Halloran Hospital was on the 1 Gm 120 day protocol, and the work done here suggests that somewhat less streptomycin resistance had been obtained

than was observed by other laboratories. The 13 per cent found resistant at the end of sixty days and the 29 per cent at ninety days might have a significant bearing on streptomycin therapy if corroborated on a much larger scale.

It has been our observation and that of others\* that there is an apparent correlation between the appearance of resistance *in vitro* and progression of tuberculous infections. In the ideal situation, therapy would be continued as long as possible without producing drug fastness. Reduction in dosage and duration of treatment has been tried. On the other hand, there is some evidence to show that intermittent injections or short series of injections may prove to give the best effect, especially as it has not been demonstrated that a high blood level of streptomycin must be maintained<sup>21, 20, 23</sup> as is the case in sulfa therapy.

*Resistance Acquired During Therapy*—During therapy the naturally resistant variants of the patient's tubercle bacilli survive and multiply thereby slowly increasing their relative proportion in the strain. In addition to selection, it seems probable that mutation in the direction of organisms with greater resistance to streptomycin also takes place<sup>24</sup> otherwise it would be difficult to explain the development of increased resistance beyond that demonstrated *in vitro* by a few colonies of the strain prior to therapy. If mutation does take place it must be due to the action of the streptomycin as the drug is the only new factor in the treatment of the patient.

*Resistance Acquired Post Therapy*—It is not without significance that in four patients (Patients 9 and 16 in Table I and 104 and 113 in Table III) of a total of twenty-one in whom pre and poststreptomycin resistances were determined the tubercle bacilli became considerably more resistant after the completion of treatment. In the light of these instances perhaps it would be advisable to wait for a number of months post therapy before final decision is made regarding the degree of resistance attained by the particular strains.

*Streptomycin Assay*—The wide range in the number of micrograms of streptomycin detected in the patient's serum after given intervals of time (16 to 64 µg, at two hours Table IV), may be accounted for by the fact that the assay method used permits determinations no closer than multiples of 4 µg (i.e., 0, 4, 8, 16, 32, 64) and also that 0.5 Gm of streptomycin was administered twice daily to all patients regardless of body weight.

*Sputum Conversion*—The absence of tubercle bacilli in smears and concentrates is one of the standards set by the National Tuberculosis Association

TABLE VI SPUTUM CONVERSION IN STREPTOMYCIN TREATED PATIENTS

INVESTIGATOR	GM / DAY	NUMBER OF DAYS	NUMBER OF CASES	CONVERSIONS		CRITERIA
				NUMBER	%	
Alto	18	120	190	8	43	69 by culture 13 by smear
Oleens	18	120	21	14	66	Culture and smear
	10	120	7	3	43	Culture and smear
San Fernando <sup>14</sup>	20	120	100	72	66	36 culture 36 smears
Williams	20	90	7	17	71	?
Hinschaw <sup>18</sup>	13	60 180	28	13	46	Culture or guinea pig

Also had 60 per cent conversions in the 1 Gm group



for the classification of a patient as "apparently arrested." The disappearance of tubercle bacilli in previously positive secretions suggests that the process has become arrested. However, there is still much confusion in the literature concerning the standards for sputum conversion.

Table VI is a compilation of the results obtained by various investigators. The criteria for conversion ranged from a stained smear to guinea pig inoculation, and the per cent of conversion from 43 to 74. The Veterans Administration<sup>17</sup> has taken a step toward clearing this situation in deciding that cultures must be negative for three successive months before classification as a sputum conversion.

On the basis of smears and concentrates, 41 per cent of the thirty-nine patients reported in this study would be classified as "apparently arrested." However, only three (8 per cent) patients could qualify as conversions by the criteria of the Veterans Administration (Patients 12, 18, and 38 in Table I).

Patient 12 was negative on three monthly cultures obtained post therapy, although the sputum did show Gaffky counts of IV, II, and III on those concentrates. This may be due to the fact that the organisms were not viable during this period.

During the early period of therapy the sputum of Patient 18 was a Gaffky VII. After forty-five days of treatment both smear and culture became negative and continued so for more than three months.

Patient 38 at the start of treatment showed a negative smear with a positive culture. After fifty days of streptomycin both were negative and remained so for the required period of time.

### CONCLUSIONS

1. The resistance to streptomycin developed by the tubercle bacillus in secretions of thirty-nine patients with pulmonary tuberculosis has been reported.

2. A regime of 1 Gm. of streptomycin daily in two doses twelve hours apart induced no resistant strains at the end of thirty days of therapy. After sixty days, 13 per cent were resistant, after ninety days, 29 per cent and after 120 days, 45 per cent.

3. In four patients (19 per cent of those tested) resistance greatly increased after the cessation of streptomycin treatment. It would therefore seem advisable to postpone final decision on the number of strains which become resistant, as a result of a streptomycin regime, until a number of months after the completion of therapy.

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# BLOOD AND CEREBROSPINAL FLUID CONCENTRATIONS OF AUREOMYCIN AFTER ORAL AND INTRAMUSCULAR ADMINISTRATION

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AUREOMYCIN\* is a recently developed antibiotic derived from *Streptomyces aureofaciens*. It has a wide antimicrobial spectrum both in vitro and in animals. Early trials in various human infections have yielded promising results. It seemed important, therefore, to determine the size of the doses and the intervals between administration which were necessary to maintain demonstrable concentrations of aureomycin in the blood. The results of such a study are reported in the present paper.

## METHOD

Since it was soon found<sup>1</sup> that aureomycin was inactivated too rapidly in vitro to determine bacteriostatic concentrations by a twenty-four hour serial dilution method analogous to the one commonly used to determine penicillin concentrations, a modification of the procedure of Dornbush<sup>2</sup> was adopted. Tryptose phosphate broth was serially diluted by halves in the usual manner with *Bacillus cereus* as the test organism and the results were read after four hours of incubation at 37° C in a water bath.

The antibiotic was administered to patients orally or intramuscularly in doses shown in Table I. Many patients received only a single dose, whereas others who were being treated for an infection had blood drawn after the first dose and at some later date (Table I).

## RESULTS

Three different dosage regimens were investigated in detail: 100 mg administered intramuscularly, and 300 and 700 mg given by mouth. When 10 mg per kilogram were given to a child, we considered it in the same category as 700 mg in an adult.

When the antibiotic was given orally, the highest concentrations were obtained in the majority of individual patients at the third hour and in a few at the sixth hour, particularly when the larger dose was employed. In only one patient was the concentration highest at the first hour. A detectable concentration of aureomycin was present at the eighth hour in all the samples of blood taken at that time. Aureomycin could be detected in twenty-six of the thirty-two specimens collected twelve hours after administration of a single dose, in four of the twelve specimens obtained eighteen hours after administration, and in eight of the thirty specimens collected at twenty-four hours. In general, the concentrations obtained from a dose of 700 mg orally were higher than those

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\*Supplied by the Lederle Laboratories Division, American Cyanamid Company.

†This strain was known as Bacillus No. 5 and was supplied by Mr. A. C. Dornbush.

TABLE I BLOOD CONCENTRATIONS OF AUREOMYCIN IN MICROGRAMS PER CUBIC CENTIMETER FOLLOWING ORAL AND INTRAMUSCULAR ADMINISTRATION

DOSE	ROUTE	PATIENT	DAY OF THERAPY	HQU CONCENTRATION MEASURED AFTER DOSE AUREOMYCIN										REMARKS	
				1	2	3	4	6	9	12	18	24			
100 mg	IM	1	Single Dose	0.29	0.50			0.25				0.06		0.06	Obese probably given subcutane- ously
		2		1.00	0.06			0.06				0.06	>0.03		
		3		0.25	0.25			0.06				0.06	>0.03		
		4		0.25	0.30			0.06				0.13	>0.03		
		5	1.00	2.00			0.06					>0.03			
		6	0.13	0.13			0.13				>0.03	>0.03			
		7	0.13	0.13			0.06				>0.03	>0.03			
		8	0.33	0.03											
		9	1.00	0.13											
		10	>0.03	>0.03											
300 mg	IM	11	DO						0.06	0.10			>0.03	>0.03	
		12	DO											>0.03	
		13	DO												
		1	DO	1.00	0.30			1.00		0.13	0.13		0.13	>0.03	
700 mg	IM	2	DO	0.13	0.30			0.30							
		3	DO	1.00	1.00										
		4	DO	>0.03	0.50			0.50		2.00		0.03	0.03		
		5	DO	0.50	2.00			1.00		>0.03		>0.03	>0.03		
300 mg	IO	6	DO	>0.03	1.00			0.25		>0.03		0.03	0.03		
		7	DO	0.24	1.00			0.30		0.25		0.25	>0.03		
		8	DO	0.06	0.50			0.25		0.03		0.13	>0.03		
		9	DO	0.13	0.50			0.25		0.13		0.13	>0.03		
		10	DO	0.25	0.25			0.50		0.13		0.13	>0.03		
		11	DO	0.13	0.25			0.50		0.13		0.13	>0.03		
		12	DO												
		13	DO												
		14	DO												
		15	DO												

IM Intramuscularly

IO by mouth

Urine collected 0 to 2 hr 64  $\mu\text{g/cc}$  or 30 mg 2 to 4 hr 198  $\mu\text{g/cc}$  or 61 mg 4 to 6 hr 198  $\mu\text{g/cc}$  or 16 mg = total 110 mg recovered in 6 hours



# MEAN BLOOD CONCENTRATIONS OF AUREOMYCIN IN PATIENTS RECEIVING VARIOUS DOSES BY ORAL AND INTRAMUSCULAR ROUTES

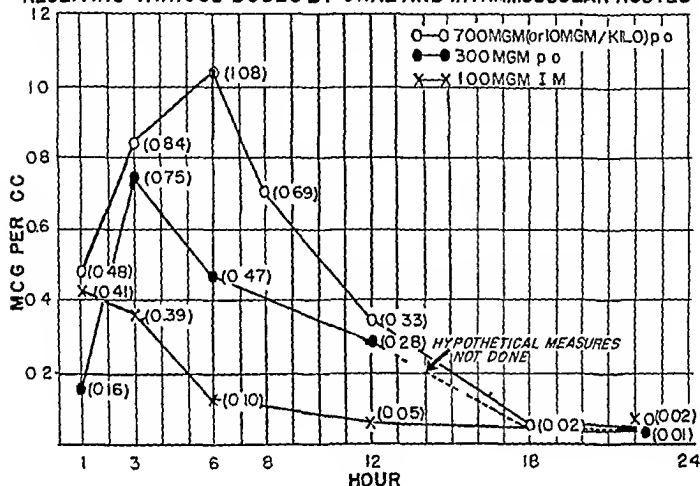


Fig 1

obtained when 300 mg were given by the same route. This is shown graphically in Fig 1, where the mean blood concentrations are plotted against time. When 100 mg were administered intramuscularly, the situation was somewhat altered. One third of the patients showed the highest concentration at the first hour, one third at the third hour, and in one third the first and third hour levels were the same. In all patients the concentration had decreased by the sixth hour. The mean curve (Fig 1) contrasts these differences sharply with those obtained after oral medication.

There are too few patients who received higher doses intramuscularly to reach any conclusions, but it appears that the concentrations were maintained for a longer period of time but were not appreciably higher than when 100 mg were given.

In the case of some of the patients who were treated over a period of several days, initial levels were determined after the first dose and these were repeated after several days. When the drug was administered at six hour intervals, the blood concentration was essentially the same for each individual patient, indicating that no appreciable amount of piling up occurred. On the other hand when the interval was shortened to three hours the levels were somewhat higher and the two patients whose blood was tested at various intervals after the start of treatment showed higher levels after several days of treatment.

Cerebrospinal fluid concentrations were measured on twelve occasions in nine patients, as shown in Table II. It seems likely that the penetration of the

antibiotic into the cerebrospinal fluid is a function both of duration of treatment and height of the blood aureomycin concentration. In the one patient with meningitis who was tested, the presence of meningeal inflammation did not seem to facilitate the passage of the antibiotic.

TABLE II SPINAL FLUID AUREOMYCIN CONCENTRATIONS IN MICROGRAMS PER CUBIC CENTIMETER COMPARED WITH BLOOD CONCENTRATIONS AND DURATION OF TREATMENT

BLOOD CONCENTRATION ( $\mu\text{G}/\text{C.C.}$ )	DAY OF TREATMENT				
	1	2	3	4	5
0.13	>0.03				
	>0.03				
0.25	>0.03				0.06
1.00	>0.03*	0.06*	0.13*		
	0.06				
2.00			0.13	0.13*	
4.00			0.13		
Unknown					0.13

\*Patient had meningitis

In one patient it was possible to collect two-hour fractional urines over the first six hours following administration of 1,000 mg. by mouth. As can be seen (Table I), about 10 per cent of the material was recovered in the urine during that period, and the urinary concentrations were quite high, being about 100 times that of the blood, or approximately what one would expect in the case of a glomerular filtrate which was not reabsorbed by the tubules.

In one patient it was possible to obtain a measure of activity of the breast milk three days after starting treatment, at a time when the blood concentration was 2.0 micrograms per cubic centimeter. No antibiotic was demonstrated.

#### DISCUSSION

While this study indicates that oral doses of 5 to 10 mg. per kilogram of body weight at six-hour intervals are sufficient to assure measurable amounts of aureomycin consistently in the blood, the optimal dosage schedule still needs to be worked out. From our data it would appear that oral administration at shorter intervals than six hours may increase the height of the level, while if a six-hour interval is maintained there is no piling up. On the other hand, if low continuous levels are desired, larger doses at longer intervals should maintain these with a minimum of disturbance to the patient. Our data suggest that the same is probably true for intramuscular administration and that large doses by this route at fairly short intervals will possibly give the highest concentrations. This has been impractical however, since even with the use of procaine as a diluent there is still too much pain following injection for routine use. Since the antibiotic is so scarce, we have not evaluated the value of a high initial dose (4 to 6 Gm.), but such a procedure may aid in reaching higher concentrations more rapidly.

A second problem remains as to what the therapeutic significance of such levels are. Some information is available which indicates that the effective blood concentration depends on the etiologic organism and that in some diseases the levels demonstrated in this paper are adequate, whereas in others they have

been insufficient. Thus the two patients with Rocky Mountain spotted fever seemed to make an excellent response to therapy, as did the two patients with pneumococcal infections. The abscess caused by *A. aerogenes* healed rapidly, but the patient with endocarditis did not respond to treatment. Moreover, none of the four patients with typhoid had what could be called a response to therapy. It would appear, therefore, that for treatment of certain infections, concentrations in the neighborhood of 0.1 to 2.0  $\mu\text{g}$  per cubic centimeter may be enough, whereas in other infections larger doses at shorter intervals will have to be tried. The good response of five other patients with Rocky Mountain spotted fever and two with pneumococcal pneumonia on whom levels were not obtained but who received equivalent dosage bears out this impression.

The data on the spinal fluid thus far obtained indicate that there is some likelihood of obtaining therapeutic concentrations in this fluid by oral or parenteral administration. That the one patient with pneumococcal meningitis whom we have treated recovered with the use of aureomycin administered by the oral route only is of importance in this regard. Details of the clinical use of this antibiotic will be reported subsequently.

#### SUMMARY AND CONCLUSIONS

1 Blood, cerebrospinal fluid, urine and milk concentrations have been measured following the administration of aureomycin orally and intramuscularly in doses of 100 to 1,000 mg in adults and equivalent amounts in children. In general the higher doses administered by either route tended to prolong the duration of demonstrable activity in the blood and to a lesser extent to increase the peak concentration.

2 Aureomycin was detected in the cerebrospinal fluid of six out of nine patients. Its concentration seemed to depend on the height and duration of the blood concentration.

3 In one patient no aureomycin was found in the milk even with a high blood concentration.

4 The drug is recoverable rather rapidly in the urine where it is found in high concentrations.

5 It is suggested that to maintain lower concentrations, large doses orally at longer intervals seem most convenient and that to obtain high concentrations shortening the interval should be effective. It is possible that a high initial dose (4 to 6 Gm) may allow higher concentrations to be reached and maintained.

6 Blood concentrations obtainable with doses of 10 mg per kilogram by mouth every six hours appear adequate for treatment of Rocky Mountain spotted fever and pneumococcal pneumonia.

We wish to thank Mrs. Joan R. Brovhill and Miss Myrtle I. Meyer for technical assistance.

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# THE ACTION OF THEPHORIN UPON HISTAMINE-INDUCED GASTRIC SECRETION IN DOGS AND ON GASTRIC ULCER FORMATION IN RATS

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CONSIDERABLE disagreement exists as to the effect of antihistaminic substances upon histamine-induced gastric secretion. This is true both of the original compounds of this type thymoxy ethyl diethylamine (929 F)<sup>1, 3</sup> and N-phenyl-N, N',N'-triethylethylenediamine (1571 F)<sup>2, 4</sup> and of the more recently developed antihistamines Benadryl<sup>4</sup> and Pyribenzamine<sup>5</sup>. The majority of investigators failed to observe any significant decrease of histamine-induced gastric secretion in dogs pretreated with antihistamines. Similar observations have been made in man<sup>8, 10</sup>. Some clinical investigators reported an increase of gastric acidity after the administration of antihistamine compounds<sup>11, 12</sup>.

The reports concerning the influence of antihistamines upon the occurrence of histamine-induced gastric ulcers in experimental animals<sup>6, 13, 15</sup> are rather unanimous. The histamine antagonists did not prevent ulcer formation.

Since Thephorin\* (2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyrimidine), which is a potent antihistamine<sup>16, 17</sup> synthesized in the Roche Research Laboratories by Dr W. Wenner and Dr J. T. Plati, differs both structurally and pharmacologically<sup>18</sup> from other histamine antagonists, it seemed desirable to study its influence upon gastric secretion and ulcer formation produced by histamine and on "spontaneous" ulcer formation.

*I The Effect of Thephorin on Gastric Secretion*—Gastric secretion was studied in three trained, unanesthetized, female dogs weighing from 10 to 12 kilograms with denervated gastric pouches (Heidenhain). Two series of experiments were carried out.

In the first series the animals received subcutaneously 0.1 mg per kilogram histamine phosphate thirty minutes after the subcutaneous injection of 20 mg per kilogram Thephorin. In a second series of experiments, 0.025 mg per kilogram of histamine phosphate was used to stimulate gastric secretion. In addition, control experiments in which the administration of Thephorin was omitted were carried out with the same doses of histamine phosphate. Each type of experiment was done in triplicate in each dog at least three days apart. Thus a total of thirty-six experiments have been performed in three dogs. The animals had been starved twenty hours prior to the experiment.

Gastric juice was collected for one hour at ten-minute intervals. Both volume and free hydrochloric acid were determined, the latter by titration using Sahli's reagent, a mixture of equal parts of 48 per cent KI and 8 per cent KIO<sub>3</sub>. In addition, the pH of each sample was measured by means of a Beckman pH

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†Deceased

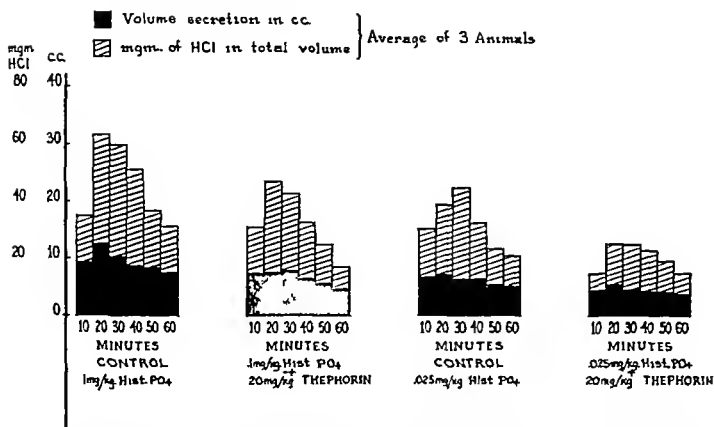


Fig. 1—Effect of Thephorin on histamine induced gastric secretion in dogs. Abscissa, time in minutes. Ordinate, solid columns, cubic centimeters of gastric juice; solid plus hatched columns, milligrams of free hydrochloric acid.

meter. The average values for each dog, from three experiments with two histamine doses each are presented in Table I which demonstrates that only the volume of secretion was reduced to a significant degree by Thephorin whereas the hydrogen ion concentration remained practically unaltered. The average decrease in volume and consequently in the quantity of free hydrochloric acid amounted to about 30 per cent regardless of the dose of histamine used. The average amount of hydrochloric acid produced by 0.025 mg per kilogram histamine phosphate is almost identical with that after 0.1 mg per kilogram histamine phosphate in the presence of 20 mg per kilogram Thephorin; thus 75 per cent of histamine has been rendered ineffective by Thephorin.

*II The Effect of Thephorin on Histamine Induced Ulcer in Rats*—Thirty Sprague Dawley male rats weighing from 280 to 340 grams (average weight 310 grams) received daily by intramuscular injection 60 mg histamine phosphate in a mixture of beeswax and mineral oil, prepared according to Varco and co-workers<sup>19</sup>. These rats had been used for a vitamin A assay several months prior to this experiment and were on a normal Rockland mouse diet. Of thirty animals used in this experiment ten received subcutaneously 20 mg per kilogram Thephorin and ten received 10 mg per kilogram atropine five hours after the histamine injection. The remaining ten rats served as controls. The treatment of the surviving animals was carried on for thirty days after which time they were killed and autopsied.

From Table II it is seen that Thephorin has a definite prophylactic effect against histamine induced ulcer formation. Eight control animals and nine of the animals pretreated with atropine died from perforated ulcer during the

TABLE I EFFECT OF THEPHORIN ON HISTAMINE INDUCED GASTRIC SECRETION IN DOGS

A 0.1 MG/KG HISTAMINE PHOSPHATE						
MINUTES	0.1 MG/KG HISTAMINE PHOSPHATE			THEPHORIN (20 MG/KG S.C.) 30 MIN. PRIOR TO HISTAMINE		
	VOLUME (CC)	FREE HCl* (MG)	pH†	VOLUME (CC)	FREE HCl* (MG)	pH†
<i>Dog 1, female, 12.4 kg</i>						
10	11.4	46.4	1.05	6.5	25.6	1.02
20	16.3	64.3	.85	8.4	60.5	.79
30	11.7	58.9	.94	8.2	42.8	.90
40	9.0	45.5	.97	6.6	31.8	.99
50	8.9	29.4	1.05	5.5	21.1	1.04
60	7.1	28.2	1.12	3.1	7.6	1.20
Total	64.4	272.7		38.5	189.6	
<i>Dog 2, female, 10.2 kg</i>						
10	9.0	28.7	.97	8.4	38.7	1.02
20	9.4	54.2	.90	6.8	35.8	.96
30	8.7	52.6	.89	6.7	45.6	.95
40	8.1	51.8	.93	5.6	29.6	.99
50	6.6	32.0	.98	5.2	23.6	1.01
60	7.2	30.2	1.04	5.8	23.0	1.05
Total	49.0	249.5		38.5	186.1	
<i>Dog 3, female, 10.7 kg</i>						
10	8.0	32.3	1.07	8.4	30.0	1.11
20	12.3	73.0	.88	8.2	47.6	.93
30	10.6	66.6	.89	9.1	50.1	.92
40	9.5	56.0	.91	7.7	37.6	.97
50	9.9	49.4	.96	6.7	31.9	1.01
60	8.8	36.0	1.04	5.5	21.7	1.05
Total	59.1	323.3		45.6	218.9	
B 0.025 MG/KG HISTAMINE PHOSPHATE						
<i>Dog 1</i>						
10	8.0	17.5	1.08	3.4	4.5	1.40
20	6.7	30.7	.92	3.5	13.6	1.09
30	6.3	32.6	.93	3.5	14.9	1.00
40	6.1	28.2	.97	3.2	16.9	1.00
50	4.7	16.9	1.06	2.9	11.2	1.08
60	4.7	14.3	1.11	3.0	8.6	1.19
Total	36.5	140.2		19.3	69.7	
<i>Dog 2</i>						
10	6.1	28.2	1.01	5.6	20.4	1.08
20	6.7	40.3	.91	4.8	24.1	.94
30	5.5	53.0	.90	4.5	22.3	.95
40	5.9	28.2	.94	3.7	17.2	.98
50	5.1	24.6	.99	4.5	17.5	1.04
60	5.3	23.2	1.05	3.7	13.4	1.05
Total	34.6	197.5		26.6	114.9	
<i>Dog 3</i>						
10	6.5	16.3	1.11	5.1	20.8	1.01
20	8.3	47.4	.88	7.1	38.7	.92
30	7.7	40.4	.93	6.6	39.1	.89
40	7.6	41.4	.96	6.0	35.2	.91
50	6.4	30.2	.99	5.8	30.6	.94
60	5.7	24.8	1.06	5.1	22.7	1.03
Total	42.2	200.5		35.7	186.7	

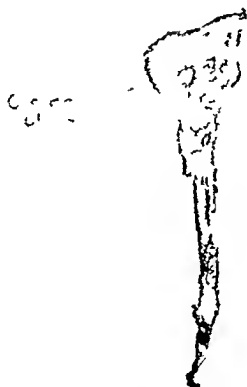
\*Calculated from titration

†The pH was averaged by converting the pH readings into normality, averaging the normality figures and reconvertng to pH

TABLE II THE EFFECT OF THEPHORIN ON ULCER FORMATION INDUCED BY THE DAILY INTRAMUSCULAR INJECTION OF 10 MG HISTAMINE PHOSPHATE IN A MIXTURE OF FISH LIVER OIL AND MINERAL OIL

DOSE, S.C. DAILY	NUMBER OF RATS SURVIVED	NUMBER OF RATS FROM ULCER DEATH ULCER	SUBSISTANT TIME OF RATS WHICH DIED FROM PERFORATION (DAYS)	TOTAL PERCENTAGE MORTALITY (%)
-	10	8	12 13 13 14 15 17 18 20	90
Thephorin, 20 mg/kg	10	3	16 18 27	33
Atropine, 10 mg/kg	10	9	13 16 16 17 17 18 27 27 27	90

(One rat which died on the eighth day from unknown cause did not show any ulcers)



Rat #26 Control

Gave of Hist Pca daily

Perforated in 13 Days

Fig. 2. Stomach of control rat with perforated ulcer after eight days from perforated ulcer after giving 10 mg of histamine phosphate daily in a mixture of fish liver oil and mineral oil.

course of the experiment. A typical lesion is illustrated in Fig. 2. A large duodenal ulcer was found in one of the two surviving control rats. In contrast only three of the rats pretreated with Thephorin succumbed to the same lesion but no ulcers were found in the surviving animals. All rats lost weight during the experimental period although all animals had free access to food and water.

III The Effect of Thephorin on Ulcer Formation in Shay Rats—Fifty male Sprague Dawley rats weighing from 130 to 150 grams prepared by the method of Shay and co-workers<sup>2</sup> were used in this experiment. Essentially, after the animals had been starved for forty-eight hours the stomach was ligated at the pylorus under light ether anesthesia. Immediately after closure of the short

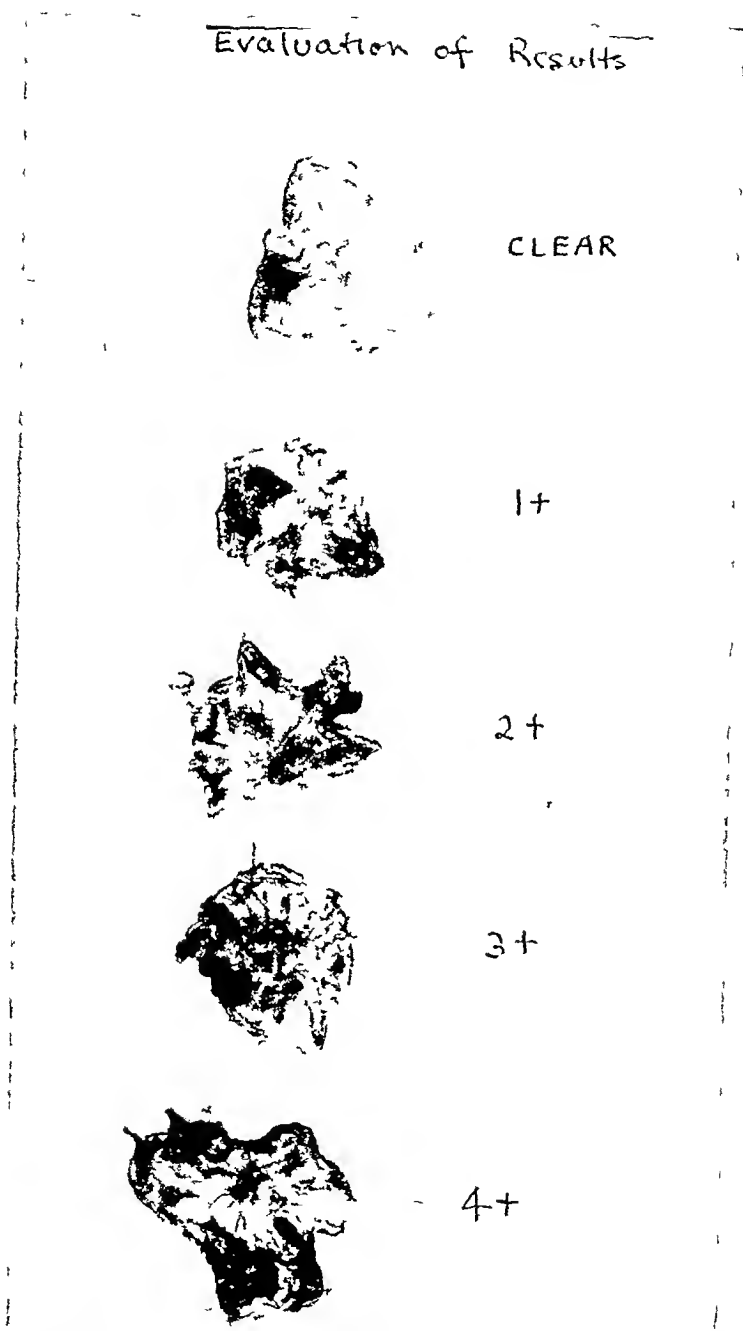


Fig 3—Evaluation of degree of ulceration. From the top down: Clear (Thephorin 20 mg per kilogram); 1+ (Thephorin 10 mg per kilogram); 2+ (atropine 10 mg per kilogram); 3+ (control); 4+ (control).

abdominal midline incision by two sutures, the rats were injected by the subcutaneous route with the substances to be tested for antiulcer effect. Doses of 20, 10, and 5 mg per kilogram of Thephorin and 10 mg per kilogram atropine were administered respectively to four groups of ten rats each. Eighteen hours

TABLE III THE ANTIGASTRIC EFFECT OF THEPHORIN IN SHAM RATS, EIGHTEEN HOURS AFTER PYLORIC LIGATION

DOSE & C	NUMBER OF PATHS LAD	DEGREE OF ULCERATION					AVERAGE DEGREE OF ULCERATION	NUMBER PERFORATED
		0	1+	2+	3+	4+		
—	10			1	1	8	3.7+	~
Thephorin, 20 mg/kg	10	1	2		1		0.5+	1
Thephorin, 10 mg/kg	10	1	4	1	1	1	1.3+	3
Thephorin, 5 mg/kg	10	1	1	6	1	1	2.0+	4
Atropine 10 mg/kg	10	3	3	2	2	1	1.3+	2

later the animals were autopsied the surviving ones being killed by ether inhalation. The degree of ulceration was evaluated as described by other investigators,<sup>9, 11</sup> scoring both the number and the size of the lesions as illustrated in Fig. 3. The results which are compiled in Table III were striking. The majority of the animals treated with 20 mg per kilogram of Thephorin were free of the typical numerous lesions found in all control animals. Only one perforation had occurred after 20 mg per kilogram Thephorin in contrast to seven in the controls. The lower doses of Thephorin had a proportionally lesser effect, 10 mg per kilogram Thephorin being approximately equivalent to 10 mg per kilogram of atropine. Although gastric juice was collected from the surviving animals

TABLE IV AVERAGE VALUES OF GASTRIC ANALYSES IN SHAM RATS FIVE HOURS AFTER PYLORIC LIGATION

	CONTROL 20 RATS	THEPHORIN (20 MG/KG 5 C) 18 RATS
Average volume	64 cc	46 cc
Average pH*	1.6	2.0
Average pepsin	9500 units	8000 units

\* The pH was averaged by converting the pH readings into normality, averaging the normality figures, and converting to pH.

and analyzed for the amount of free hydrochloric acid the data are useless for comparison since the stomach contents of the surviving control animals had a relatively high pH caused by the buffering action of blood from the lesions. To gather information about possible differences in acidity and the amount of pepsin between control animals and rats pretreated with 20 mg per kilogram Thephorin, a five hour experiment was carried out. Since practically no ulceration had occurred within this period the true hydrogen ion concentration values of the gastric juice could not be ascertained. The average results for volume, pH, and pepsin concentration—the latter determined by the method of Kleiner<sup>22</sup>—are recorded in Table IV. The figures obtained from the animals pretreated with 20 mg per kilogram Thephorin are somewhat lower than those from the control animals, but hardly significantly.

#### DISCUSSION

Thephorin has a definite effect in reducing the gastric secretion induced in dogs by histamine. This particularly antihistaminic action of Thephorin is less marked than its action against other important effects of histamine. In a series of experiments the volume of gastric juice secreted was reduced by 30 per cent which corresponds to the inactivation of 75 per cent of the administered histamine.

Contrary to reports in the literature<sup>14</sup> we succeeded in producing gastric ulcers in rats by repeated administration of histamine. The incidence of ulcer formation was markedly decreased after Thephoin in contrast to control animals and rats pretreated with atropine.

The fact that the rats had been used previously for a vitamin A assay may have been an important factor for the occurrence of ulcers. It has been reported<sup>23, 24</sup> that vitamin A deficiency per se leads to gastric ulcer formation in rats. The possibility whether a low vitamin A intake earlier in life is a predisposing factor for the production of ulcers by histamine in rats has to be considered. This problem is now under investigation and preliminary experiments seem to indicate that high doses of vitamin A have a prophylactic effect against histamine-induced ulcer formation.

Quite different were the results on ulcer formation in Shay rats. Under these experimental conditions both Thephoin and atropine were equally effective in protecting rats against ulcer formation. Considering the small difference in hydrogen-ion concentration and in the amount of pepsin between controls and treated animals, the antiulcer action of Thephoin cannot be explained by its inhibiting effect on the secretion of gastric juice. It has been shown<sup>14</sup> that vagotomy can prevent ulcer formation in Shay rats in the presence of a high acid concentration. The antiulcer effect of atropine is explained by its peripheral vagal blocking action, but this is unlikely to be the case with Thephoin since it was demonstrated that its anticholinergic effects are relatively weak as compared with atropine.<sup>17, 18</sup> The possibility has to be considered that a central mechanism is involved in the prophylactic action of Thephoin which is effective in the presence of a relatively high acid concentration. It is generally assumed that the degree of acidity, although a major factor in the genesis of peptic ulcer, is not the sole cause.<sup>25</sup> The evidence accumulated in these studies suggests that the action of Thephoin represents a chemical vagotomy.

It seems unlikely that histamine is the causative agent of the lesion occurring in Shay rats since atropine has a marked antiulcer effect under these experimental conditions but does not prevent histamine-induced ulcer formation.

#### SUMMARY

The antiulcer effect of Thephoin in Shay rats was striking. The evidence available suggests that the effect may be considered as chemical vagotomy.

Thephoin prevented to a marked degree gastric ulcer formation in rats produced by repeated histamine administration. Atropine was ineffective.

Thephoin reduced histamine-induced gastric secretion in dogs with Heidenham pouches by about 30 per cent.

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## THE EFFECTS OF INTRAVENOUSLY ADMINISTERED HISTAMINE ON THE PERIPHERAL CIRCULATION IN MAN

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THE use of continuous intravenous administration of histamine as a therapeutic agent in certain diseases made us interested in a study of its effects on the peripheral circulation in the patients receiving the drug. Benson and Horton<sup>1</sup> studied the effects of continuous intravenous injection of histamine on the blood pressure and heart rate in patients with multiple sclerosis. They did not obtain a significant change in the systolic blood pressure, but the diastolic blood pressure showed an average fall of 17 mm. of mercury with a range of fall of 9 to 27 mm. of mercury below the control value. Within ten minutes after administration of the drug had been stopped, the pressures returned to within 3 mm. of mercury of their original levels. The heart rate increased an average of thirty beats per minute, with a range of increase from twenty-three to thirty-nine beats per minute. The heart rate also returned quickly to control levels after administration of histamine had been stopped.

In an excellent study of the systemic effects of histamine in man, Weiss and associates<sup>2</sup> reported that histamine is promptly converted into inactive substances in the human body. They found the minimal effective amount of histamine base in man to be about 0.003 mg. per minute. In observations of two hours' duration histamine up to toxic doses failed to produce any lowering of systolic arterial blood pressure, but the diastolic pressure showed only a tendency to fall and in many instances it also remained unaltered. They stated that the effect of histamine on the cutaneous blood vessels is not uniform. The most characteristic effect is dilatation of the venules, independent of the dilator effect on the arterioles. In some cases the evanescent flush and lack of elevation of the cutaneous temperature suggested a failure, even of toxic doses, to dilate the arterioles. Sometimes larger doses are needed to dilate the arterioles than those that dilate the venules. The intravenous infusion of 0.02 to 0.03 mg. of histamine base per minute increased the minute cardiac output about 20 per cent over the control value and was accompanied by a slight reduction in stroke volume. A slight but distinct increase in the mean velocity of blood flow was noted.

Haimeir and Harris,<sup>3</sup> and also Cairnel,<sup>4</sup> stated that histamine produced dilatation of the skin arterioles in man. Haimeir and Harris noted an increase in heart rate of twenty beats per minute but were unable to detect plethysmographically any increase in blood flow to the limb as a whole in human subjects.

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under the influence of histamine. Thermocouples, however, showed a rise in skin temperature which was interpreted as indicating that the blood is flowing more quickly through the skin vessels. When histamine was given intravenously they noted that the effects were complete in three to four minutes. One twentieth milligram of histamine hydrochloride increased cutaneous blood flow. Reddening of the skin and rise in skin temperature were definite. The systolic and diastolic blood pressure fell slightly and there was some increase in heart rate. Carrier reported that the application of histamine to the base of the fingernail in normal human beings in a dilution from 1 1,000 to 1 10,000 caused widening of the capillaries and acceleration of capillary blood flow.

In summarizing the evidence Best and McHenry<sup>1</sup> stated that small doses of histamine have little if any effect on the heart but somewhat larger doses increase the force and rate of the heartbeat in most species.

Anrep<sup>2</sup> reported that histamine dilated the coronaries in the human heart lung preparation.

In a study of the vasodilator action of histamine and its physiologic significance, Burn and Dale<sup>3</sup> explained the difference in reaction to histamine observed in various species of animals as being due to the variation of the level at which the constrictor effect of histamine on the central portion of the vascular tree changes to a dilator effect on the peripheral portion. In the dog the constrictor effect changes to a dilator effect at the level of the small visible arteries. In the cat the constrictor effect is present even at the level of the arterioles but mostly in the capillaries. In the rodents, the constrictor effect is pushed so far to the periphery that its change to a dilator effect escapes detection.

From his work on histamine shock Rich<sup>4</sup> concluded that histamine exerts a local dilator effect on capillaries and on the smallest arterioles and venules which border the capillary system. The circulatory failure in histamine shock is due to dilatation of the peripheral vascular bed.

From the work of Dale and associates<sup>1, 5, 11</sup> and Weiss and associates<sup>2</sup> it is suggested that there probably exists a shift in the locus of action of histamine as the evolutionary tree is ascended. Arterial constriction may be the predominant effect in rodents. In the cat there is a slight constriction of the arterioles, but the capillaries are dilated. In the dog and the monkey both arterioles and capillaries dilate. In man also the arterioles and the capillaries of the skin and of the brain dilate.

Since as the literature convincingly indicates the action of histamine varies in different species it is hazardous to interpret its action in man by observations obtained from animal experimentation. This makes it obvious that in order to obtain information on the action of histamine in man the observations should be made on man. This study deals with the effects of histamine on the peripheral circulation in human beings who were given histamine for therapeutic purposes and who volunteered for the investigation. The clinical observations and the laboratory data were obtained simultaneously on the

same subjects, in order to avoid species or individual differences, and under the same conditions, in order to avoid as many as possible of the factors that might introduce variations

#### METHODS

Human subjects who were receiving histamine intravenously by infusion over a period of about two hours for therapeutic purposes volunteered for this study. Before the observations were started, the patient was asked to lie quietly on a comfortable bed for about thirty minutes in a constant temperature room at 77° F with a humidity of about 40 per cent. Blood flow studies on all four extremities were made by means of the venous occlusion plethysmograph with a compensating spirometer recorder.<sup>12</sup> The arm plethysmographs included the hand, wrist, forearm, and part of the arm up to 2 inches (about 5 cm) above the olecranon process. The leg plethysmographs included the foot, ankle, and leg up to 1 inch (about 2.5 cm) below the tibial tuberosity.

The cutaneous temperatures were recorded galvanometrically by means of thermocouples applied to the forehead, to the skin over the right and left deltoid muscles, and over the right and left quadriceps femoris muscles. All five thermocouples were kept in position throughout each period of observation (more than two hours), and the temperatures were recorded as required at intervals.

The heart rate and systolic and diastolic blood pressure were recorded by the usual clinical procedures.

Histamine diphosphate was administered to each patient intravenously by continuous infusion in a concentration of 1:250,000 in physiologic saline solution at successive rates of 0.004, 0.008, 0.016, 0.020, and 0.024 mg of histamine diphosphate per minute respectively. The duration of infusion at each rate was twenty minutes. Control values for skin temperatures, heart rate, blood pressure, and blood flow were established before the infusion of histamine was started. All the observations were repeatedly taken at regular intervals thereafter for each of the periods of infusion at each of the infusion rates and for five to fifteen minutes after the infusion had been stopped.

#### RESULTS

Table I presents the percentage change in blood flow in the four extremities of subjects that were given histamine diphosphate intravenously by continuous infusion at the various rates specified. It can be stated generally that the magnitude of increase in blood flow of the upper extremities is greater than that of the lower extremities and that in both cases the increase is roughly proportional to the dose of histamine administered; namely, the larger the dose, the greater the increase in blood flow. In a few cases, especially when small doses of histamine were given, there was a transient reduction in blood flow in one or more extremities. Because Patients 8 and 21 could not tolerate higher dosage, the infusion of histamine was stopped at the dose of 0.016 mg per minute. Five minutes after the intravenous infusion of histamine had been stopped, the augmentation in blood flow was very much reduced. Evidently, as indicated from this work and from others,<sup>2, 13, 14</sup> considerable amounts of histamine are rapidly inactivated in the body.

The changes in the systolic blood pressure produced by the intravenous infusion of the amounts of histamine used in the study were insignificant, but the diastolic pressure showed a consistent drop of 10 to 15 mm of mercury. However, soon after the infusion of histamine had been stopped, the blood pressure returned to the control level.

TABLE I PERCENTAGE CHANGE OVER CONTROL BLOOD FLOW IN THE EXTREMITIES DUE TO INTRAVENOUS ADMINISTRATION OF HISTAMINE

PATIENT	EXTREMITY	MINUTES OF HISTAMINE 1. USED PER MINUTE					5 MIN AFTER STOPPING OF INFUSION
		0.002	0.008	0.016	0.020	0.024	
1	Left arm						-50
	Right arm	-11	-14	-165		-166	-181
	Left leg	-17	-1	-3		-15	-82
	Right leg	-1	-1	-32		-15	-47
5	Left arm						-36
	Right arm	-2	-1	-84			-3
	Left leg	-	10	17			-37
	Right leg	-	-	-8			-7
13	Left arm						-24
	Right arm	16	-	-8		-73	-36
	Left leg	-11	-1	-56		-160	-75
	Right leg	-4	11	-6		-47	-27
14	Left arm						-17
	Right arm	1	-		-26		-17
	Left leg	-4	6		-3		-24
	Right leg	-	10		-5		-26
15	Left arm						-34
	Right arm	-	-	-128	-157		-47
	Left leg	-1	-66	-126	-12		-137
	Right leg	10	-	-40	-117		-7
16	Left arm	-3	-6		-100		-
	Right arm						-52
	Left leg	-1	67		-107		-84
	Right leg	-3	-44		-50		-42
17	Left arm	-18	-3	-22	-317	-203	-28
	Right arm						-111
	Left leg	-20	-4	-38	-2	-70	-16
	Right leg	-2	-	-36	-64	-80	-10
18	Left arm	-12	-12	-100	-66		-67
	Right arm						-26
	Left leg	-2	-10	-5	-12		-3
	Right leg	21	17	-	-13		-2
19	Left arm	-30	-103	-	-300		-16
	Right arm						-71
	Left leg	-10	-6	-14	-8		-7
	Right leg	-6	-22	-20	-80		-15
20	Left arm	6	-17	-104	-117	-106	-1
	Right arm						-10
	Left leg	-52	-11	-20	-63	-87	-6
	Right leg	0	-20	-17	-	-19	-17
21	Left arm	-50	-20	-2			
	Right arm						
	Left leg	-11	-12	-52			
	Right leg	-13	-54	-23			
22	Left arm	-57	-42	-67	-70		
	Right arm						
	Left leg	-6	-58	-84	-58		
	Right leg	-34	-25	-10	-18		
Average	Left arm	-10	-4	-146	-10	-24	-4
	Right arm	-15	-28	-92	-6	-120	-31
	Left leg	-20	-22	-58	-81	-76	-40
	Right leg	-12	-10	-26	-2	-25	-17

Percentage change in blood flow

TABLE II AVERAGE CHANGE IN CUTANEOUS TEMPERATURE DURING INFUSION OF HISTAMINE (TEN CASES)

THERMOCOUPLE AREA	CUTANEOUS TEMPERATURE BEFORE HISTAMINE	AMOUNT OF HISTAMINE GIVEN (MG PER MIN)				
		0.004	0.008	0.016	0.020	0.024*
Forehead	34.1° C	0.2° C	0.2° C	0.3° C	0.2° C	0.2° C
Right shoulder	32.4	0	0.1	0.5	1.0	2.2
Left shoulder	32.8	0.1	0.4	1.1	1.3	1.8
Right thigh	31.3	0.4	0.6	1.4	1.8	3.1
Left thigh	31.7	0.5	0.6	1.2	2.1	3.6

\*Three cases only

In eight subjects the heart rate was taken during infusion of the drug at the various rates. The average increases in heart rate over the control value were six beats per minute for the infusion rate of 0.004 mg of histamine per minute, six beats for the rate of 0.008 mg per minute, nineteen beats for the rate of 0.016 mg per minute, and twenty-three beats for the rate of 0.020 mg per minute. Within ten minutes after the infusion of histamine had been stopped, the pulse rate practically returned to the control value.

Table II shows the average rises over the control temperatures produced during the intravenous infusion of histamine. It can be stated generally that, with the exception of the forehead, the greater the rate of infusion of histamine, the greater the rise in cutaneous temperature. Soon after the infusion of histamine had been stopped, the cutaneous temperature began to subside toward the control level.

As seen in Table II, the changes in skin temperature were greater over the lower extremities than over the shoulders and forehead. This is attributable to the relatively much greater perspiration over the forehead and shoulders than over the mid-region of the lower extremities. We believe that the cooling due to the accumulated fluid secreted by the sweat glands under the thermocouples accounts for the relatively lower temperature recorded from the skin over the forehead and shoulders.

## COMMENT

When it was repeatedly observed that the flushing and increase in blood flow were greater in the upper parts of the body and in the upper extremities than in the lower extremities, three possible explanations came to mind. The first two and more probable ones were as follows: (1) The upper parts of the body (face, neck, and arms) are usually more directly exposed to the environmental factors and consequently more intrinsic histamine and histamine-like substances are continuously liberated. This endogenous histamine supplemented the effect of infused histamine and gave findings of greater magnitude in the upper extremities than in the protected lower extremities. (2) The presence of a vasomotor gradient, vasoconstriction being least in the face, progressively increasing downward, and becoming highest in the toes, may account for the differences in degree of flushing and redness, which were greatest in the face and were progressively less till they were almost unnoticeable over the legs during the administration of histamine.

Fundamental differences in the vasomotor reactions of the hands and feet have been observed. Pickering and Hess<sup>1</sup> reported that in normal subjects the vasodilatation in response to warming of the body becomes evident in the fingers earlier than it does in the toes. They concluded that the delayed response of the toes as compared with the fingers, is attributable not to a difference in time but to a difference in intensity of the vasomotor relaxation in upper and lower extremities. As a result of warming of the body, loss of vasoconstrictor tone in the upper extremities is complete while in the lower extremities it is incomplete.

Horton and associates<sup>1</sup> noted prompt rise in the surface temperature of the fingers in patients given typhoid vaccine intravenously, whereas there was a distinct lag in the rise in surface temperature of the toes as compared with that of the fingers.

A distinct vasomotor gradient was demonstrated in normal subjects with reference to the face, hands and feet after administration of alcohol. The maximal degree of vasodilatation was in the face with decreasing degrees in the hands and feet. Regardless of the vasodilator agent used the rise in surface temperature appeared first in the face then in the fingers and later in the toes. The degree of vasoconstriction is greater in the toes than in the fingers. The upright posture in man may account for the greater degree of vasoconstriction in the lower extremities as compared with that of the upper. The difference in intensity of vasoconstriction in the toes and fingers may not be evident in terms of surface temperature but is easily demonstrated when vasodilator agents are administered.

A third possibility that because the arm veins were used for infusion the upper parts of the body received more histamine before it was exposed to the destructive enzymes of the blood and tissues was disproved and eliminated when we gave the infusion of histamine several times into the leg veins instead of the arms and still the flush and increase in blood flow were greater in the upper parts of the body and upper extremities than in the lower extremities.

#### SUMMARY

Intravenous administration of histamine di-phosphate in a concentration of 1:250,000 in saline solution at successive rates of 0.004, 0.008, 0.016, 0.020 and 0.024 mg. per minute respectively produced cutaneous vasodilatation which appeared first over the face and neck of the patient and gradually extended downward over the upper extremities and thorax, reaching the lower extremities only toward the end of the period of observation when the higher rates of infusion were used. There was a definite increase in skin temperature and in heart rate and a slight decrease in diastolic blood pressure.

The blood flow in the four extremities gradually increased in proportion to the dosage used until at the highest rate of infusion of 0.024 mg. of histamine per minute the average increase in blood flow over the control values was 244 per cent in the left arm, 120 per cent in the right arm, 76 per cent in the left

leg, and 28 per cent in the right leg. However, five minutes after the infusion of histamine had been stopped, the increase of blood flow averaged only 46 per cent in each of the arms, 40 per cent in the left leg, and 14 per cent in the right leg. The changes in skin temperature, blood flow, heart rate, and blood pressure gradually subsided, and the values returned toward the control level shortly after the infusion of histamine had been stopped.

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## PHOTOELECTRIC DETERMINATION OF ARTERIAL OXYGEN SATURATION IN MAN

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THE Matthes,<sup>1</sup> Millikan<sup>2</sup> and Goldie<sup>3</sup> oximeters have greatly facilitated the study of changes in arterial oxygen saturation in man. However, since these instruments must be preset to known arterial saturation values they cannot be conveniently used in patients who may have arterial hypoxia, nor can they be used for the actual determination of arterial oxygen saturation. Furthermore, extensive calibration studies with the Millikan compensated circuit oximeter carried out in this laboratory have yielded such variable results that it has been concluded that oximeter saturation readings are of only qualitative value in indicating changes in arterial oxygen saturation. Consequently studies have been carried out in an attempt to eliminate or minimize these shortcomings of the instrument.

A device will be described herein which can be used to measure the absolute value of, and follow continuously the changes of the arterial oxygen saturation from a pickup unit attached to the pump of the human ear. This is accomplished by means of a photoelectric earpiece which allows simultaneous measurement of the transmission of red and near infrared light through either the normal heat flushed ear or the bloodless ear. Then by calculation, the light transmission of the blood alone in these spectral regions can be determined and in turn the percentage oxygen saturation of this blood content can be derived.

### OPERATION AND CONSTRUCTION OF THE INSTRUMENT

Although it was originally assumed that the Millikan oximeter responded to transmitted light in the region of the red and the green,<sup>2</sup> it has been pointed out<sup>4,5</sup> that whole blood films of approximately 1 mm. depth transmit practically no light of a wave length shorter than 600 millimicrons.<sup>7</sup> The normal human ear has similar characteristics.<sup>8</sup> Therefore the operation of the oximeter is based on differences in transmission of light by oxygenated and reduced hemoglobin which occur in the red and near infrared rather than in the red and green portions of the spectrum as originally supposed.

The Millikan oximeter earpiece contains two barrier layer iron selenium photoelectric cells, each covered by a specific Wratten gelatin optical filter. Assuming a constant light source in the earpiece the amount of light transmitted

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leg, and 28 per cent in the right leg. However, five minutes after the infusion of histamine had been stopped, the increase of blood flow averaged only 46 per cent in each of the arms, 40 per cent in the left leg, and 14 per cent in the right leg. The changes in skin temperature, blood flow, heart rate, and blood pressure gradually subsided, and the values returned toward the control level shortly after the infusion of histamine had been stopped.

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## PHOTOELECTRIC DETERMINATION OF ARTERIAL OXYGEN SATURATION IN MAN

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THE Matthes<sup>1</sup>, Millikan<sup>2</sup> and Colditz<sup>3</sup> oximeters have greatly facilitated the study of changes in arterial oxygen saturation in man. However, since these instruments must be preset to known arterial saturation values they cannot be conveniently used in patients who may have arterial hypoxia, nor can they be used for the actual determination of arterial oxygen saturation. Furthermore, extensive calibration studies with the Millikan compensated current oximeter carried out in this laboratory have yielded such variable results<sup>4</sup> that it has been concluded that oximeter saturation readings are of only qualitative value in indicating changes in arterial oxygen saturation. Consequently, studies have been carried out in an attempt to eliminate or minimize these shortcomings of the instrument.

A device will be described herein which can be used to measure the absolute value of and follow continuously the changes of the arterial oxygen saturation from a pickup unit attached to the puny of the human ear. This is accomplished by means of a photoelectric earpiece which allows simultaneous measurement of the transmission of red and near infrared light through either the normal heat flushed ear or the bloodless ear. Then by calculation, the light transmission of the blood alone in these spectral regions can be determined and in turn the percentage oxygen saturation of this blood content can be derived.

### OPERATION AND CONSTRUCTION OF THE INSTRUMENT

Although it was originally assumed that the Millikan oximeter responded to transmitted light in the region of the red and the green,<sup>2</sup> it has been pointed out<sup>5,6</sup> that whole blood films of approximately 1 mm. depth transmit practically no light of a wave length shorter than 600 millimicrons.<sup>7</sup> The normal human ear has similar characteristics.<sup>8</sup> Therefore the operation of the oximeter is based on differences in transmission of light by oxygenated and reduced hemoglobin which occur in the red and near infrared rather than in the red and green portions of the spectrum as originally supposed.

The Millikan oximeter earpiece contains two barrier layer iron selenum photoelectric cells, each covered by a specific Wratten gelatin optical filter. Assuming a constant light source in the earpiece, the amount of light transmitted

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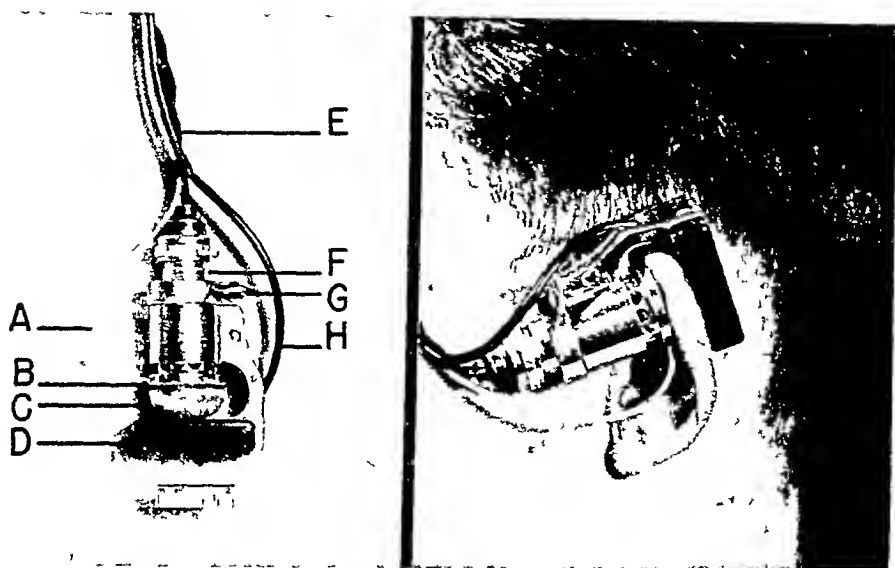


Fig 1—Modified oximeter earpiece. A, inlet to pressure capsule B, pressure capsule C, rubber diaphragm of pressure capsule (capsule inflated to 10 mm of mercury) D housing for photoelectric cells E leads to light bulb F adjustable carrier of light bulb and pressure capsule G, adjustable clamp to fix position of light bulb and pressure capsule H leads to photocells

through the pinna of the ear and the filters to the photoelectric cells is a function of the fixed characteristics of the respective filters, the amount of ear tissue, and the amount and state of oxygenation of the blood in the path of light

One of the photocells is covered by a green Wratten 61N filter, which transmits light in the spectral region from 480 to 600  $m\mu$  and in the region above 750 millimicrons. This cell, which has been called the "green cell," actually responds to light in the near infrared. This is due to the fact that the ear absorbs practically all light of a wave length shorter than 600  $m\mu$  and the response of the photocell falls practically to zero for light of a wave length greater than 850 millimicrons. Therefore this cell (the "infrared cell") is activated only by light of a wave length of approximately 800 millimicrons. Since oxygenated and reduced hemoglobin transmit light of this wave length to practically the same degree,<sup>6, 10</sup> the output of the infrared cell is a function of the amount of ear tissue and blood in the optical path and is independent of the degree of oxygenation of this blood.

The other photocell is covered by a red Wratten 29F filter, which transmits light of wave lengths greater than 600 millimicrons. The percentage transmission of red light (600 to 750  $m\mu$ ) is very different for oxygenated and for reduced hemoglobin<sup>11, 12</sup>. Hence the output of this cell (the "red cell") is a function of the amount of ear tissue and *both the amount and the degree of oxygenation* of the blood in the path of the light. If the ear tissue and amount of blood are constant, the output of the red cell is a function of the oxygen saturation of the blood interposed in the optical path of the earpiece.<sup>1, 12</sup>

In the Millikan oximeter the amount of ear tissue and blood in the optical path is compensated for by adjusting the instrument to indicate a known value of saturation (usually 98 or 100 per cent when the subject is breathing air or 100 per cent oxygen) while the earpiece is in place on the ear on which it is to be used. Variations in the amount of blood in the optical path are then compensated for automatically by balancing the output of the infrared cell against the output of the red cell.

The instrument to be described is based on the same spectral transmission characteristics of hemoglobin as is the Millikan oximeter. It differs however, in the type of earpiece, infrared filter and electrical circuits used.

The earpiece (Fig. 1) differs from the earpiece of the Millikan oximeter in that the light source instead of being fixed in position is incorporated into an adjustable cylindrical housing designed so that it can be fixed securely in

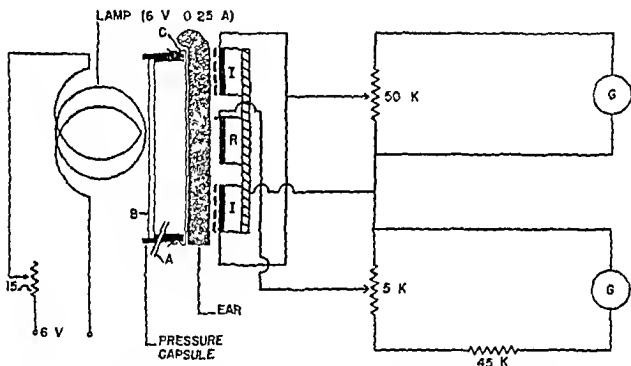


Fig. 2.—Circuit diagram of modified oximeter. 1 inlet to pressure capsule. E glass disk. C rubber diaphragm. G galvanometers (50 cm scale, coil resistance 3800 ohms, optimal damping resistance 50 000 ohms, sensitivity 0.0006 microampere per millimeter deflection, period 4 seconds). I iron selenium photoelectric cells covered by a Wratten 84 infrared filter (dashed line). R iron selenium photoelectric cell covered by a Wratten 9F red filter (dotted line). 50 50 000 ohm potentiometer control for infrared cell. 5 K 5 000 ohm potentiometer control for red cell.

the desired position relative to the ear. The end of the light housing which comes in contact with the ear contains an airtight chamber, the pressure capsule. The surface of the pressure capsule toward the light bulb is of glass, while the surface toward the ear is covered with a thin translucent rubber dam, thus absorption by the capsule of the light being transmitted from the light bulb to the ear is relatively small. A 1 cm length of No. 22 gauge hypodermic tubing serves as an air inlet to the pressure capsule and is connected to a hand bulb and mercury manometer by means of a suitable length of vinyl tubing. When the earpiece is placed on the ear the unit housing the light is adjusted so that the end (covered with rubber dam) fits snugly but not tightly against the front surface of the pinna. Thus when the pressure capsule is inflated to arterial

occlusive pressures (200 mm of mercury) the pinna is compressed between the rubber diaphragm and the flat, front surface of the photocell housing so that the blood is squeezed from the portion of the ear within the optical pathway of the earpiece. The photocells are covered with a mask containing a circular opening 12 mm in diameter, which is 8 mm less than the diameter of the surface of the pressure capsule. Masking of the photocells in this manner assures that all light reaching the cells is transmitted through a portion of the ear which can be efficiently pressurized by the capsule.

An infrared Wratten 87 gelatin filter is used to cover the infrared cell in place of the Wratten 61N filter used in the ordinary oximeter earpiece. Falsely high values for the infrared transmission of the bloodless ear are obtained if the 61N filter is used, since it has been found that the "bloodless ear" transmits significant amounts of light in the green spectral region transmitted by the 61N filter.

The outputs of the red and the infrared cells are recorded separately by means of galvanometers<sup>\*</sup> which have a sensitivity such that 0.0006 microampere produces a deflection of 1.0 millimeter. A schematic diagram of the circuit used is shown in Fig. 2.

#### PROCEDURE

The earpiece is placed on an optical filter† (E 100) and allowed to become warm. The infrared and red cell outputs are adjusted by regulating their respective variable resistors to produce equal galvanometer deflections (usually 50 to 70 mm depending on the anticipated optical density of the ear). Readings are then taken in succession on the E 100, E O, and E R test filters,‡ after which the earpiece is placed on the ear. Galvanometer readings are recorded at intervals of sixty seconds until a constant value is reached, indicating that the ear has become fully flushed. Usually this occurs within ten minutes. The pressure capsule is then inflated to 200 mm of mercury and readings are recorded at intervals of thirty seconds for five minutes. The pressure is then released and readings are continued for an additional period of five minutes or until the completion of some observational procedure. Readings are usually taken at intervals of ten seconds during withdrawal of arterial blood samples and at intervals of thirty seconds during the administration of low oxygen mixtures. If the observational procedure is of more than thirty minutes' duration, the "bloodless ear readings" (pressure capsule inflated to 200 mm of mercury) are redetermined at the end of the period of observation. Readings on the optical filters are repeated at the end of the procedure in order to be certain that the response of the device has remained stable throughout the period of observation.

\*Rubicon box galvanometers (catalogue No. 3415) with a coil resistance of 1500 ohms, a critical damping resistance of 40,000 ohms, and a period of 5 to 6 seconds were used. A single galvanometer can be used by incorporating a double pole double throw switch so that either the red or the infrared circuit can be connected individually to the single galvanometer. A galvanometer specially designed for this instrument is available (Rubicon Company) consisting of two galvanometer elements housed in a single cabinet with a single direct (visual) reading scale 20 cm. in length.

†The E-100 filter is composed of the following materials in the order in which they are named:

- 1 One piece of glass 21 mm in thickness ground on the inner surface with No. 3F carborundum grit.
- 2 One layer of tracing paper (Albanene tracing paper 195L-12 Keuffel and Esser Company, Hoboken, N. J.)
- 3 One Wratten 29F filter
- 4 Three layers of tracing paper
- 5 One Wratten 29F filter
- 6 One layer of tracing paper
- 7 One plain glass slide measuring 11 mm in thickness.

‡The E-O, E-100, and E-R filters were constructed empirically to simulate the transmission characteristics for near infrared and red light of an average bloodless ear and an ear flushed with 90 and with 30 per cent saturated blood respectively. The composition of the E-R filter is identical to that of the E-100 filter except that one of the 29F filters is replaced by a Wratten 70 filter. The E-O filter consists of an 0.50 Wratten neutral density filter interposed between the same glass components as used for the E-100 and E-R filters.

## CALCULATIONS

The amount of light absorbed by the portion of the ear interposed between the constant light source and the photocells of the earpiece is an inverse function of the logarithm of the galvanometer deflection produced by the output of the photocells. Furthermore, the total light absorption by this portion of the ear is an additive effect of the light absorbed by the ear tissue proper and by the blood contained in this ear tissue. Consequently the amount of light absorption by the blood contained in this ear tissue is a function of the difference between the light absorption of the bloodless ear and the light absorption of the blood containing ear.

Therefore a value which is a function of the absorption of infrared light by the blood ( $IR_b$ ) can be obtained by the following equation

$$IR_b = \log II - \log IR_s = \log \frac{IR}{IR_s}$$

in which  $IR$  is the galvanometer deflection produced by the infrared cell when the ear is rendered bloodless by the pressure capsule and  $IR_s$  is the galvanometer deflection produced by the infrared cell when the ear is in the normal blood containing state.

Similarly a value ( $R_b$ ) which is a function of the absorption of red light by the blood interposed in the optical path of the earpiece can be obtained by the expression  $R_b = \log \frac{R}{R_s}$  in which  $R$  is the galvanometer deflection produced by the red cell when the ear is rendered bloodless and  $R_s$  is the galvanometer deflection obtained from the blood containing ear.

The absorption of light by blood in the near infrared is a function of the total amount of hemoglobin contained in the blood. Likewise absorption of red light by this blood is a function of the amount of oxyhemoglobin and total hemoglobin. Therefore, the ratio  $R_b/IR_b$  is a function of the oxygen saturation of the blood interposed in the optical path of the earpiece. Since this blood is rendered arterial by the increase in blood flow through the ear produced by the heat of the earpiece,<sup>†</sup> the expression  $R_b/IR_b$  gives a value which is a function of the percentage saturation of arterial blood with oxygen.

The exact nature of the relationship between  $R_b/IR_b$  and arterial oxygen saturation may be determined experimentally and a calibration curve which will allow oxygen saturation to be determined from  $R_b/IR_b$  ratios may be constructed.

## METHODS

The calibration and range of error of the instrument have been studied in three ways:

1. The ratio  $R_b/IR_b$  has been calculated from the readings of the instrument obtained when recording from thirty five ears of twenty three normal subjects (age range, 5 through 68 years) while they were breathing room air or 100 per cent oxygen. The values of  $R_b/IR_b$  obtained for each ear have been plotted against the arterial oxygen saturation on the assumption that the arterial oxygen saturation of these subjects was 97.9† and 100 per

The mathematical expression of this relationship can be derived on the basis that Beer's law applies to the transmission characteristics of red and infrared light by hemoglobin in circulating whole blood assuming (1) that the response of the infrared cell is completely independent of the oxygen saturation of the transilluminated blood.

$$S = \frac{100 K}{K-1} (\log R/R_s - \log IR/IR_s) - \frac{100 I}{1-I}$$

or (2) that the response of the infrared cell is not completely independent of the degree of oxygen saturation of the transilluminated blood.

$S = 100 (K \log R/R_s - K \log IR/IR_s) - [(1-I) \log IR/IR_s - (K-1) \log R/R_s]$  in which  $S$  is the per cent oxygen saturation of the transilluminated blood,  $I$ ,  $K$ ,  $1$ ,  $1/I$ ,  $1/K$  and  $K-1$  are constants, the absorption coefficients of total oxygenated and reduced hemoglobin for red and infrared light and  $R$ ,  $R_s$ ,  $IR$  and  $IR_s$  are defined in the text.

The equations were derived by Mr R. J. Shaw, Fellow in Biology, Los Mayo Foundation.

† Average value based on Van Slyke analyses of arterial blood sample obtained from twenty nine resting subjects breathing air (altitude 1040 feet). Extreme values .44 to .101 per cent.

cent when breathing air and oxygen respectively. The average values obtained have been used to establish the calibration curve of the instrument in the region from 95 to 100 per cent arterial oxygen saturation (Fig 3)

2 By the use of an indwelling arterial needle,<sup>13</sup> blood samples have been obtained from the radial artery of five normal white and five normal Negro subjects after periods of two and a half to ten minutes of breathing each of the following seven gas mixtures

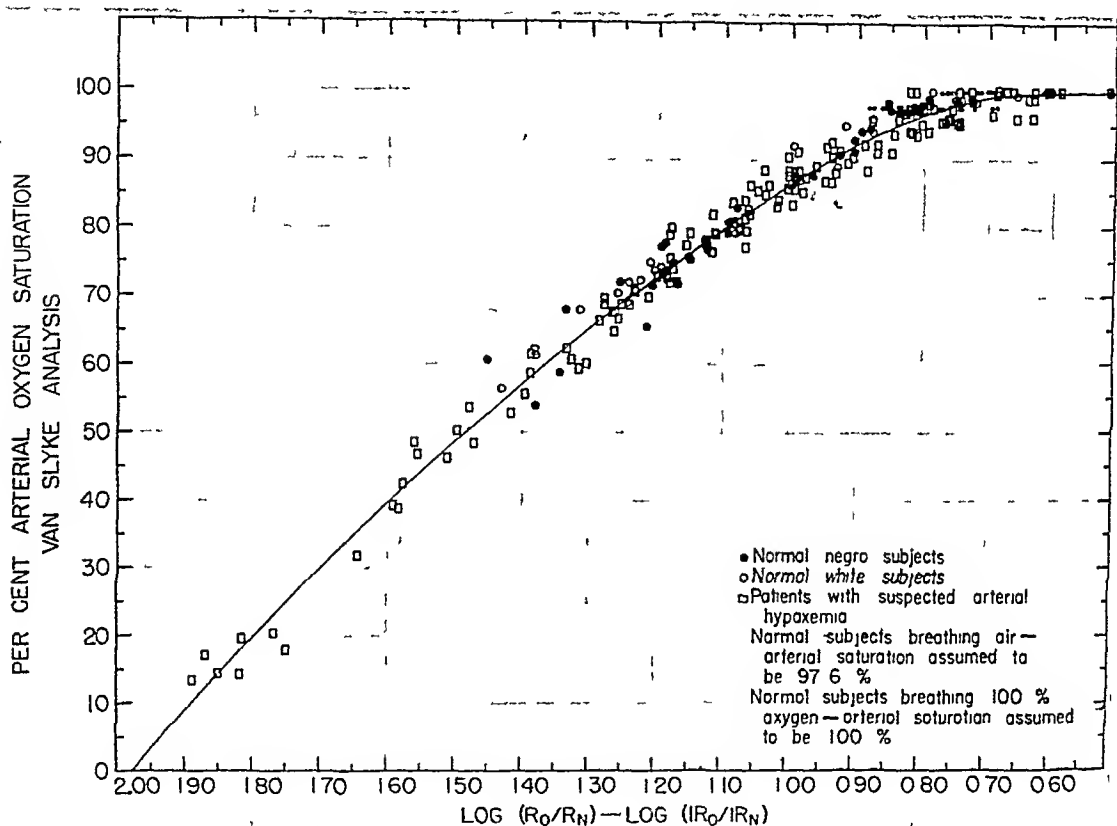


Fig 3.—Empirical calibration curve of modified oximeter earpiece (No 1) for photoelectric determination of absolute values of arterial oxygen saturation in man. Based on 211 simultaneous photoelectric and Van Slyke determinations of arterial oxygen saturation

air, 100 per cent oxygen, and 16, 14, 12, 10, and 8 per cent oxygen in nitrogen. Instrumental readings were recorded throughout the period of withdrawal of each of sixty-two arterial blood samples. These readings were averaged and the average value of  $R_N/I_{R_N}$  for each sampling period was calculated. The values obtained have been plotted against the arterial oxygen saturation as determined by Van Slyke analyses<sup>14</sup> of the simultaneously withdrawn arterial blood samples (Fig 3)

3 Similar methods have been used to obtain instrumental readings during withdrawal of 150 arterial blood samples from a series of forty-five patients suspected to have arterial hypoxemia. Arterial hypoxemia in these cases was a result either of congenital cardiac anomalies or of acquired respiratory abnormalities. Polycythemia was present in the majority. The average values of  $R_N/I_{R_N}$  calculated from the instrumental readings obtained over the period of arterial sampling have been plotted against the arterial oxygen saturations determined by the Van Slyke analysis of the blood (Fig 3)

TABLE I AVERAGE CALIBRATION DATA OF PHOTOELECTRIC EARPIECE FOR DETERMINATION OF ABSOLUTE VALUES OF PERCENTAGE SATURATION OF ARTERIAL BLOOD WITH OXYGEN

SUBJECT	NUM BER OF SUB JECTS	AGE IN YEARS	GALVANOMETER DEFLECTION (MM.)						PER CENT O SATURA TION OF ARTERIAL BLOOD			DIFFERENCE IN CAPACITY SATURATION POINTS (R-A)	OXYGEN CAPACITY (CC O PER 100 CC ARTERIAL BLOOD)
			BLOOMFIELD'S EM		NORMAN FAY		100 (R / IR) LOG (IR / IR + R)	PHOTO ELECTRIC ESTIMA TION OF PERCENT SATURA TION OF BLOOD (P)	DIFFERENCE IN CAPACITY SATURATION POINTS (R-A)				
			RED CELL R	INFRARED CELL IR	RED (PFI) R	INFRARED CELL IR							
Normal subjects breathing 100% O <sub>2</sub>	3	8	56 (31119)	82 (60103)	60 (399)	40 (4091)	0.700 (0.204)	94.9 (94.6100)	0.5 (-0.71025)	19.1 (17.5224)			
Normal subjects breathing air	1	10	87 (31119)	53 (60103)	5 (399)	41 (4091)	0.800 (0.204)	91.8 (94.6100)	1.0 (-1.01010)	19.1 (17.5224)			
Normal subjects breathing 10% O <sub>2</sub>	10	10	53 (31113)	84 (60103)	42 (399)	58 (4091)	0.800 (0.204)	91.8 (94.6100)	0.9 (-1.11020)	19.1 (17.5224)			
Normal subjects breathing 33% O <sub>2</sub>	2	2	71 (31103)	5 (60103)	41 (399)	4 (4091)	1.000 (0.00120)	80.2 (72.286)	1.2 (-0.41025)	19.1 (18.4203)			
Normal subjects breathing 12% O <sub>2</sub>	9	9	81 (31113)	84 (60103)	40 (399)	40 (4091)	1.111 (0.92156)	81.1 (68.8118)	2.1 (-0.61063)	19.2 (17.7225)			
Normal subjects breathing 10% O <sub>2</sub>	10	10	54 (31113)	86 (60103)	43 (399)	43 (4091)	1.210 (1.12128)	71.5 (67.4770)	1.2 (-0.71049)	19.1 (17.8224)			
Normal subjects breathing 8% O <sub>2</sub>	10	10	51 (31113)	89 (60103)	40 (399)	40 (4091)	1.340 (1.1144)	62.2 (57.5691)	0.9 (-0.71076)	19.1 (17.8224)			
Patients suspected to have arterial hypoxemia	45	150	98 (36113)	91 (60103)	47 (399)	46 (4091)	1.054 (0.91192)	77.6 (72.100)	0.5 (-0.910125)	25.0 (16.5358)			

The oxygen saturation was calculated after correction of the oxygen content by subtracting the amount of oxygen estimated to be in physical solution (0.3 and 2.0 volumes per 100 c.c. for normal subjects breathing air and oxygen respectively).

†Determinations were made on both ears of nine of the 9 subjects making a total of twenty nine ears studied while subjects were breathing 100 per cent oxygen. Average age of subjects was 30 years (5 through 68 years) six of the subjects were Negroes.

‡Figures in parentheses are extreme values.

§Determinations were made on both ears of twelve of these subjects.

¶Arterial samples obtained under various conditions including rest walking on a treadmill and breathing gas mixtures containing from 8 to 100 per cent oxygen. Average age of patients was 7 years (3 through 55 years).



## RESULTS

The plot of the calculated values of  $R_R/IR_D$  against the simultaneous estimations of arterial oxygen saturation by Van Slyke analysis (Fig 3) does not reveal significant differences in the data obtained from the three groups of subjects studied. Therefore, all of these data have been used as a basis for constructing a calibration curve for the instrument. The empirical calibration curve (Fig 3) was drawn as the closest visual fit to the 211 experimental points.

When this calibration line was utilized, the photoelectrically determined values of arterial oxygen saturation of twenty normal subjects when breathing 100 per cent oxygen ranged from 94.6 per cent to 100 per cent. These subjects ranged in age from 5 through 68 years. Six were Negroes (Table I). The average arterial oxygen saturation as determined by Van Slyke analyses<sup>14</sup> of thirty-five arterial samples obtained from twenty normal subjects while they were breathing 100 per cent oxygen was 99.1 per cent and the range of values was from 97.3 to 101.1 per cent.\*

The photoelectric determinations of arterial oxygen saturation of twenty-three subjects when breathing air ranged from 92.4 to 99.7 per cent (Table I). The average arterial oxygen saturation as determined by Van Slyke analyses<sup>13</sup> of forty-six arterial samples obtained from twenty-nine normal subjects while breathing air (altitude 1,040 feet) was 97.9 per cent. The range of values was from 94.1 to 101.0 per cent.

At values of arterial saturation between 15 and 100 per cent, the standard deviation of the differences between 211 simultaneous photoelectric and Van Slyke determinations of arterial oxygen saturation was 2.9 percentage saturation points. This implies a likelihood of 95 per cent that determinations of arterial oxygen saturation by this photoelectric method will be within 5.8 per cent saturation of that obtained by Van Slyke analysis of a simultaneously withdrawn arterial sample. The range of variation was not appreciably different for white and Negro subjects and for patients with variable degrees of polycythemia (Table I)†.

## COMMENT

Photoelectric determination of the oxygen saturation of blood samples *in vivo* has been successfully carried out by several investigators.<sup>8-11, 17-23</sup> Photoelectric determination of *changes* in blood oxygen saturation also has been accomplished in unopened arteries and veins and in the intact pinna of the human ear.<sup>1-3, 12, 24</sup>

The present report represents to the authors' knowledge, the first description of the determination of absolute values of arterial oxygen saturation in the intact pinna of the human ear. Squire<sup>24</sup> in 1940 described a method for the

\*When 100 per cent oxygen was breathed for periods of ten minutes the quantity of oxygen<sup>12</sup> carried in physical solution was assumed to be 2.0 cc per 100 cubic centimeters. Therefore the oxygen contents obtained under these conditions were corrected for this factor by subtracting the constant figure 2.0. Much of the variability in the arterial oxygen saturations obtained when breathing oxygen probably results from variations in the quantity of physically dissolved oxygen and hence erroneous corrections for this factor.<sup>12</sup>

†It is of interest that the instrument predicted the arterial saturation satisfactorily (1) when used on a patient whose blood contained 0.75 Gm of sulfhemoglobin per 100 cc (2) when used over a scrubbed and blistered area on an ear and (3) when used on the ear a fold of skin on the neck and a fold of skin on the thorax of a patient who had dermatolysis.

determination of the quantity of blood in the web of the hand and its degree of oxygenation. He did not however use the method to determine arterial oxygen saturation and did not present data as to the accuracy of the method for determining the degree of oxygen saturation of the blood contained in the web of the hand.

The accuracy of the method described herein appears adequate for intermittent or continuous clinical determination of arterial oxygen saturation and should obviate in many cases the necessity of arterial punctures for this purpose. Multiple determinations on individual subjects have indicated that when large errors do occur they tend to be systematic for that subject. This is substantiated by the fact that nine of the fifteen instances in which the difference between simultaneous photoelectric and Van Slyke determinations exceeded 5 per cent occurred in three subjects.

The accuracy of the instrument was limited in part by the accuracy of the galvanometer readings. The instrument must be set so that the galvanometer deflections obtained with the bloodless ear are on scale (that is 120 mm with the galvanometers used). When the instrument was adjusted in this manner the average red cell deflection was 60 mm when the subjects' blood was 100 per cent saturated and was 40 mm when the subjects' saturation was decreased to an average value of 62 per cent by breathing 8 per cent oxygen. Under these circumstances an error of 1 mm in reading the galvanometer deflection produced by the red cell may result in an error of approximately 3 per cent in the predicted value of arterial saturation.\*

The accuracy of the photoelectric estimations of arterial oxygen saturation was not disturbed by the subjects walking on a treadmill at 1.7 miles per hour,<sup>5</sup> consequently the method would appear to be readily applicable to the study of arterial oxygen saturation during various cardiovascular respiratory function tests<sup>5, 6</sup> and during operative procedures.

Previous oximeters have not been calibrated to indicate arterial saturations of less than 50 per cent. The present instrument however operates satisfactorily at very low levels of arterial oxygen saturation. Seven simultaneous photoelectric and Van Slyke determinations of arterial oxygen saturation have been made in four patients at levels of arterial oxygen saturation ranging from 15 to 21 per cent (Fig. 3). It is of considerable physiologic interest that three of these patients were conscious and rational and able to walk on a treadmill at 1.7 miles per hour with levels of arterial oxygen saturation which in normal persons would produce loss of consciousness within a few seconds.

Since the instrument was found to perform satisfactorily on very deeply pigmented ears (Negroes) it is probable that the method can be applied to the determination of oxygen saturation of the blood contained in any human or animal tissue which will allow application of a pickup unit and an incorporated pressure capsule so that the interposed tissue can be successfully transilluminated and subjected to arterial occlusive pressures.

Appreciable errors in saturation produced by the error in reading the galvanometer can be eliminated by use of compensated shunt boxes to reduce the galvanometer sensitivity during recording of the bloodless ear readings or by use of Rubicon type L galvanometers (catalogue No. 3615 Rubicon Company Philadelphia Pa.) which deflect linearly over a scale of 100 millimeters.

It should be pointed out that all of the data reported herein were collected by the same oximeter operator, using the same earpiece, control box, galvanometers, and voltage on the earpiece light source. Every effort was made to keep all conditions which might possibly affect the operation of the oximeter as constant as possible. Therefore the degree of applicability of these data to other earpieces and less rigidly controlled conditions is not certain.

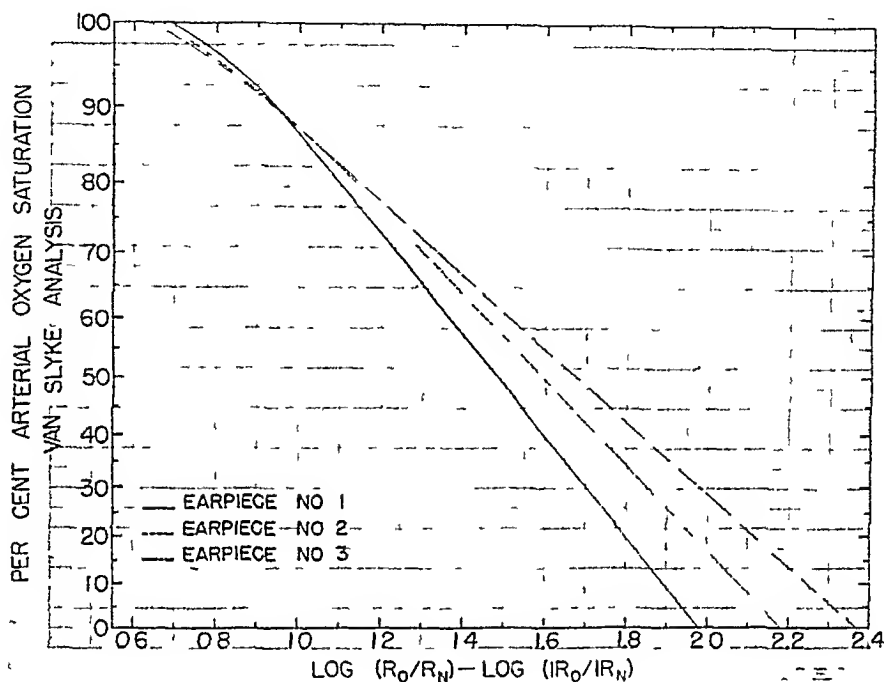


Fig 4—Empirical calibration curves of modified oximeter earpieces Nos 1, 2, and 3 based on 211, 105, and 155 simultaneous photoelectric and Van Slyke determinations respectively of arterial oxygen saturation. Plotting the oxygen saturation (ordinate) as an inverse log scale extending from 5 to 10 causes the calibration curves to approach straight lines at arterial saturations of less than 90 per cent.

Sufficient data have been collected to establish the empirical calibration curves of two other direct reading earpieces. These earpieces differed from the one used for the present study in details of design and in the use of a double thickness SSA infrared filter in place of the S7 filter. The data were collected by several different oximeter operators (technicians) under a variety of conditions. The empirical calibration curves of these earpieces were found to be significantly different from the curve of the earpiece used herein (Fig 4), and the results were somewhat more variable (Fig 5).

The following factors have been determined to be responsible at least in part for the increased variability: (1) The pressure capsule must be fitted sufficiently snugly against the front surface of the ear so that no shift in position of the ear relative to the photocells can occur when the pressure capsule is inflated. (2) Changes in the voltage across the light source may produce significant changes in the calibration curves of the earpiece. (3) The orientation and

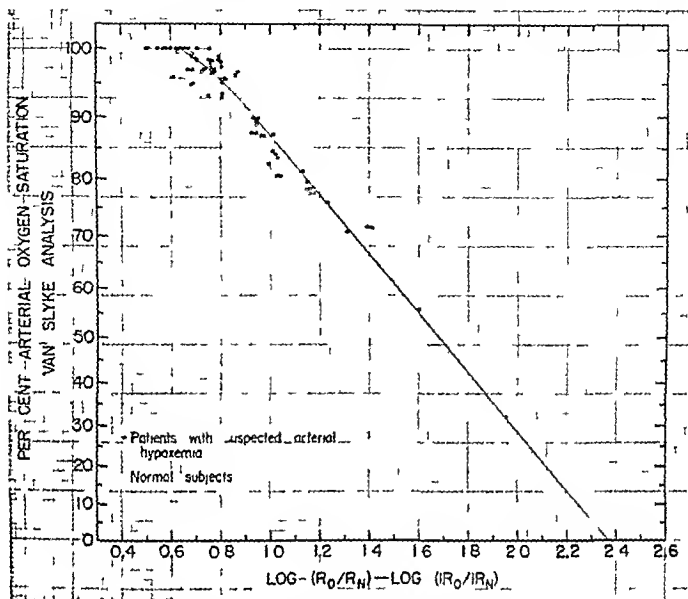


Fig. 5.—Empirical calibration curve of modified oximeter earpiece No 3 based on 15 simultaneous photo electric and Van Slyke determinations of arterial oxygen saturation. The differences between the gasometric and photoelectric values were less than plus minus 6.0 per cent saturation in 94 per cent of instances. Eight of the ten determinations which exceeded this range were obtained on two subjects. The open and solid symbols are based on determinations made with the earpiece equipped with a pressure capsule with an external diameter of 15 and 1 mm. respectively.

the distance of the filament of the light source from the photocells may alter the results obtained. A new earpiece has been designed which considerably facilitates the control of these factors.

Other than the possible variability between different earpieces, the chief disadvantage of the modified oximeter is that a calculation on the basis of four galvanometer readings reference to  $\log_{10}$  arithmetic tables and references to the empirical calibration curve are required in order to obtain the actual value of the arterial saturation.

Use of a nomogram (Fig. 6) relating infrared and red galvanometer readings directly to arterial saturation greatly expedites this procedure. If there are 100 divisions in the galvanometer scale (Fig. 7) and if during the initial bloodless ear reading the output of the photocells is adjusted so as to indicate 100, the normal (nonpressurized) ear readings can be obtained directly in terms of per cent of the bloodless ear readings. Arterial saturation then can be obtained by direct reference to the nomogram without the necessity of intermediary calculations.

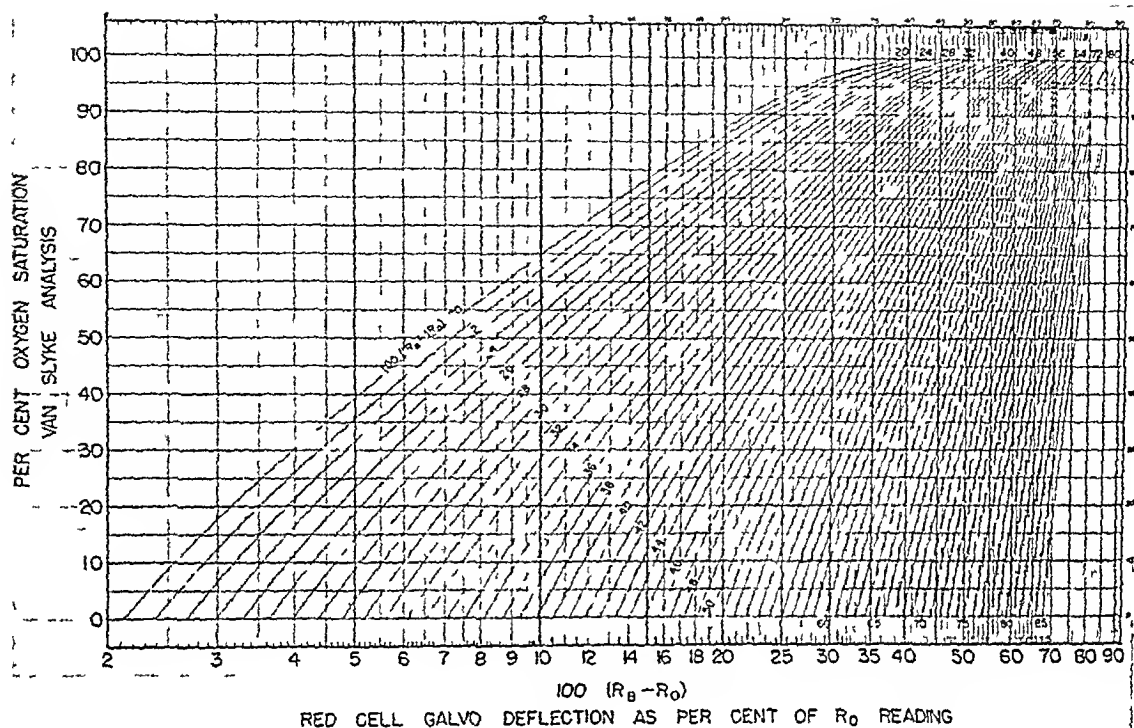


Fig. 6—Calibration nomogram derived from empirical calibration curve of modified oximeter carpiece No. 3. The nomogram can be used most conveniently if the red and infrared cell galvanometer scales are constructed so as to have 100 divisions for full scale deflection (Fig. 7). If the instrument is adjusted so that the galvanometer readings are 100 in the bloodless state ( $R$  and  $IR$ ) then the galvanometer readings for the blood-containing state ( $R_x$  and  $IR_x$ ) will read directly in terms of percentage of the  $R_0$  and  $IR$  readings. Consequently the saturation value can be read directly from the nomogram without recourse to calculation.

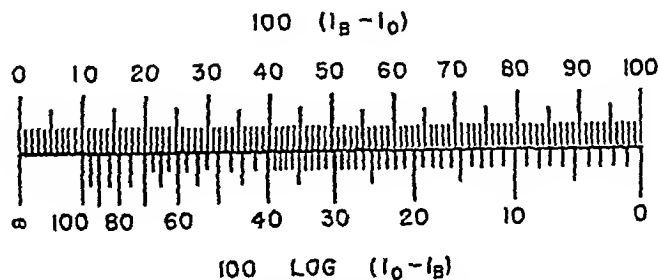


Fig. 7—Galvanometer scale for modified oximeter. When these types of scale are used the sensitivities of the red and infrared circuits should be adjusted so that full scale galvanometer readings (0 and 100) are obtained in the bloodless state ( $P_0$  and  $IR$ ). Then the bottom scale galvanometer readings for the blood-containing state ( $R_x$  and  $IR_x$ ) will be directly in terms of the log ratio ( $\log \frac{IR}{IR_x}$  or  $\frac{R}{R_x}$ ) and will be in terms of percentage of the bloodless reading ( $100 R/P$  or  $100 IR_x/IR$ ) for the top scale.

If such a homogeneity is not available the calculations required can be reduced to a single operation (division) if the bloodless ear readings are adjusted to the full scale galvanometer deflection and the galvanometer scale is calibrated to indicate deflections as the logarithm of the quotient of the full scale galvanometer deflection divided by the deflection\* (Fig 7). Then the relative

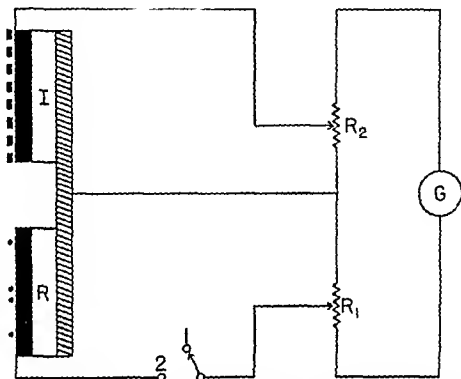


Fig 8—Circuit diagram for converting the modified oximeter to a single scale device recording directly in terms of blood oxygen saturation.

I Infrared photocell (dashed line represents double thickness Wratten 88A infrared filter)

R Red photocell (dotted line represents Wratten 82F red filter)

$R_2$  Potentiometer control for infrared cell

$R_1$  Potentiometer control for red cell

G Galvanometer ( $R_1 + R_2 =$  optimal damping resistance)

1 Control switch position for adjusting sensitivity of infrared cell (a earpiece on flushed ear)

2 Control switch position for adjusting sensitivity of red cell (b earpiece on bloodless [pressurized] ear)

After adjustments 1 and 2 are completed the galvanometer deflections produced in switch position 2 are a function of the oxygen saturation of the blood in the flushed ear.

arterial saturation (log ratio) can be obtained by dividing the red galvanometer reading by the infrared reading, and in turn the absolute value of saturation can be derived by reference to the empirical calibration curve of the earpiece being used.

Electronic means can be devised to modify this instrument so as to indicate the arterial saturation directly on a single galvanometer scale. One of the simplest methods would be to incorporate the red and infrared cells into a differential bucking circuit as suggested by Matthes<sup>1</sup>. A circuit of this type has been devised (Fig 9); it is somewhat similar to that used by Millikan<sup>3</sup>, Hemingway and Taylor<sup>4</sup>, and Hartman and associates<sup>5</sup>. Calculations based on calibration data reported herein indicate that if this circuit operated according to the theory of its design the variability of the single scale method of operation would be approximately 60 per cent greater than with the double galvanometer method which has been used. However, if the single scale circuit is to operate as designed, it is necessary that the output of the photocells be independent of the

variations in their external load resistance encountered under operating conditions. Actually this has been found not to be the case.<sup>30</sup>

### SUMMARY

An instrument is described for the photoelectric determination of absolute values of arterial oxygen saturation in the intact pinna of the human ear.

The instrument has been calibrated by determinations made on twenty-three normal subjects while breathing air and 100 per cent oxygen and by comparing the photoelectric values with values for the saturation of arterial blood with oxygen determined by Van Slyke analysis of 211 simultaneously withdrawn arterial blood samples.

In the range from 15 to 100 per cent saturation of arterial blood with oxygen the standard deviation of the difference between the photoelectric and Van Slyke determinations of arterial oxygen saturation was 2.9 percentage saturation points. Ninety-five per cent of the 275 photoelectric determinations were within plus-minus 5 per cent saturation of values based on direct gasometric analysis.

The accuracy of the procedure does not appear to be affected by age, race, sex, or blood hemoglobin content of the subjects on whom the instrument is used.

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## ISOLATION OF HERPES SIMPLEX VIRUS ON THE CHORIOALLANTOIC MEMBRANE

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WITH THE TECHNICAL ASSISTANCE OF LILLIAN T. SCHERMFRHORN

SINCE the work of Gruter<sup>1a</sup> (1913) and of Lowenstein<sup>1b</sup> (1919) the standard technique for the isolation of herpes simplex virus has consisted of inoculating the suspected material onto the scarified cornea of a rabbit. More recently, several workers<sup>2</sup> have shown that the virus grows well in the embryonated egg and that this medium may be used for primary isolation of the virus. However, no data appear to be available on the comparative efficiency of the two methods for this purpose. As the egg technique is simpler and cheaper than the standard technique, it seemed profitable to determine whether it were equally efficient. The results of parallel isolations, using the two techniques, are herewith presented, together with certain information concerning the carrying-out of the technique, the standardization of the antibiotics used, and the type of diluent that has been found useful in the routine handling of the virus.

### EXPERIMENTAL

*Comparison of Chorioallantoic Membrane (CAM) and Rabbit Cornea*—Using the standardized techniques to be described later in this paper, material from twenty-seven patients with vesicular lesions of the skin or lesions of the mucous membranes was inoculated simultaneously on eggs and on rabbit corneas. The results shown in Table I indicate no significant superiority of either method. Subsequent studies in this laboratory, using the embryonated egg as the sole means of isolating herpes simplex virus, have given gratifying results. Since that comparative study was made over a year ago, virus has been isolated by this method from thirty-four patients with various clinical manifestations of herpes simplex infection. In addition, it has been possible to get a quantitative count of the amount of virus in herpes vesicle fluid simply by measuring the fluid in a calibrated capillary tube before diluting it with buffer and antibiotic mixture. From the dilution and the number of plaques on the membrane, one can easily compute the virus content of vesicle fluid at various stages of its development.

### TECHNIQUES

In order to compare the chorioallantoic membrane and rabbit cornea as media for the isolation of herpes simplex, the specimen, such as saliva or skin washings, was divided into two parts, one being inoculated onto both corneas of a rabbit, and the other, after suitable preparation, into eggs.

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TABLE I. ISOLATION OF HERPES SIMPLEX VIRUS ON THE RABBIT CORNEA VS CHORIOALLANTIC MEMBRANE

	RABBIT CORNEAS	CAM
Number of specimens tested	27	7
Number of virus isolations	17	16
Number negative	10	11

*Rabbit Cornea Inoculation*—Both corners of young four to six pound rabbits were scarified with a sharp scalpel under ether anesthesia and the material to be tested for virus was dropped into the conjunctival sac and rubbed on the corner with a cotton swab. If the rabbit developed keratoconjunctivitis one of the eyes was removed for histologic section and if the rabbit died part of the



Fig. 1.—Rabbit cornea epithelial cells infected with herpes simplex. Characteristic intranuclear homogeneous inclusion bodies are surrounded by dense margined chromatin. Hematoxylin and eosin ( $\times 900$ ).

brain was used for serial passage and part was fixed for histologic section. It is well to emphasize at this point that the corner must be taken at the stage of early congestion, erythema and photophobia in order to demonstrate the maximal number of inclusion bodies.

*Corneal Response and Identification*—In specimens free of virus the corner is fully healed within twenty-four hours whereas the corner infected with herpes simplex shows progressive corneal opacities together with successive injection, laceration and purulent exudate of the bulbar and palpebral conjunctiva. The identity of the virus in exudates shown to be fice of bacteria was established by (1) demonstration of typical intranuclear inclusion bodies in the epithelium

of cornea or nictitating membrane (Fig 1), or in the brain when encephalitis and death occurred, (2) subsequent intracerebral inoculation with a known virulent strain of herpes virus, to test for immunity in those animals which recovered

#### PREPARATION OF SPECIMENS FOR EGG INOCULATION

*Suitable Diluent*—It has been shown frequently that the herpes simplex virus is quite unstable when suspended in physiologic sodium chloride solution, but is more stable when nutrient broth or serum is used as a diluent

Both serum and broth have certain technical disadvantages as satisfactory diluents for clinical specimens. A search was made, therefore, for a simple solution suitable for receiving specimens and for storage of strains of virus. The quantitative survival of several egg-adapted strains of herpes simplex virus was tested after storage up to one week at room temperature in 10 per cent normal rabbit serum, Tyrode's solution, nutrient broth, physiologic saline solution, and phosphate buffered saline at pH 7.2 containing 0.5 per cent gelatin. Our results confirmed previous findings that physiologic saline solution caused marked loss of virus within one to four hours' exposure. In all the other solutions the virus was stable for four hours or more, but deteriorated slowly thereafter. Storage at 4° C or at -20° C prolonged the viability of virus stored in all solutions except physiologic saline. Because of its ease of preparation and lack of antigenicity, the buffered saline-gelatin solution was adopted as the standard diluting fluid in our work<sup>2d</sup>. Nutrient broth also occasionally was employed.

*The Use of Antibiotics*—Clinical specimens that are normally free of bacteria (i.e., spinal fluid, blood) can be inoculated onto the egg membranes directly, other specimens normally contaminated with bacteria, such as saliva or vesicle fluid, usually kill the embryos from bacterial infection unless specially treated. It has been demonstrated<sup>3</sup> that sputum can be rendered safe for inoculation into eggs by treatment with penicillin and streptomycin. In relation to the isolation of herpes virus on the chorioallantoic membrane, the amount of antibiotic to be used is limited on the upper side by its toxicity both for the virus and for the chorioallantoic membrane, but must be sufficient to kill the bacteria usually encountered.

Contaminated specimens were collected on a sterile swab or capillary pipette and emulsified in 0.9 ml. of buffered saline-gelatin solution or nutrient broth. To this tube was added 0.1 ml. of a stock solution of penicillin and streptomycin. This mixture was allowed to stand ten to thirty minutes at room temperature before inoculation into the egg membranes.

*Toxicity of Antibiotics for the Chorioallantoic Membrane*—It was found that penicillin caused no appreciable changes in the chorioallantoic membrane when used in concentrations up to 10,000 units per milliliter, whereas streptomycin caused nonspecific opacities, marked thickening and edema, if applied in a concentration of 1,000 µg per milliliter or greater.

*Toxicity of Antibiotics for Herpes Virus*—Various concentrations of the two antibiotics mixed with a chorioallantoic membrane suspension of herpes simplex were tested for quantitative survival of the virus at intervals up to

twenty four hours after mixing. Ten thousand units per milliliter of penicillin and 250 µg per milliliter of streptomycin did not reduce the virus plaque count.

**Toxicity of Antibiotic Mixture on Chorioallantoic Membrane and Herpes Virus**—On the basis of the toxicity studies just described, a mixture of antibiotics was selected which gave a final concentration after being added to the virus containing specimen, of 500 units of penicillin and 100 µg of streptomycin. Many tests with this mixture showed that it was nontoxic for the chorioallantoic membrane, and that it did not appreciably diminish the amount of viable virus when tested against a laboratory strain of egg adapted HF virus.

**Effect of Antibiotic Mixture on Bacterial and Yeast Contaminants**—Most samples of sputum were rendered bacteriologically sterile by exposure to this antibiotic mixture for ten to thirty minutes. The antibiotic mixture was found to be still effective against bacterial contaminants after storage in a vaccine bottle at 4° C for up to two months. In the few failures observed the eggs were shown to be contaminated by yeasts or molds.

TABLE II ADDITIVE EFFECT OF MIXED ANTIBIOTICS FOR A BACTERIUM AND A YEAST FREQUENTLY FOUND IN THE MOUTH

ANTIBIOTIC	YEAST EFFECTIVE CONCENTRATION AGAINST	
	STREPTOCOCCI*	C. ALBICANS†
Penicillin	0.026 u/ml	>500 u/ml
Streptomycin	100 u/ml	>100 u/ml
Crystal violet‡	1:200 000	1:100 000
Streptomycin	10 u/ml	— — —
+ Crystal violet	1:500 000	— — —
Penicillin	0.003 u/ml	25 u/ml
+ Streptomycin	0.0006 u/ml	5 u/ml
+ Crystal violet	1:7 500 000 000	1:1 000 000

In vitro tests by the Hammelkamp method for penicillin assay.

†In vitro tests with various amounts of antibiotic or mixtures added to 0.5 per cent glucose broth containing a standard inoculum of *C. albicans*. Subcultures made to glucose broth to determine the fungicidal concentration.

‡Coleman and Bell certified.

Since crystal violet is a well known fungicide, dilutions of crystal violet were tested for toxicity to the chorioallantoic membrane to the virus of herpes simplex, and to *Candida albicans* in order to see if it could be used to control the fungi occasionally encountered in sputum.<sup>4</sup> On the basis of these studies, crystal violet was added to the antibiotic mixture to make a final concentration in the clinical specimen of 1 to 50 000. Although the addition of crystal violet caused a precipitate to form (the crystal violet base apparently precipitates with the penicillin anion), the bactericidal properties of the mixture were enhanced rather than reduced as shown in Table II. This mixture sterilized sputum which was naturally or artificially contaminated with *C. albicans* and permitted the isolation of herpes simplex when present. In spite of its efficacy, crystal violet has not been used routinely because in our experience, contaminating fungi were seldom encountered.

**Inoculation of Eggs**—Two to four eggs prepared by the "false air sac technique"<sup>2a</sup> each were inoculated through the hole over the false air sac with 0.05

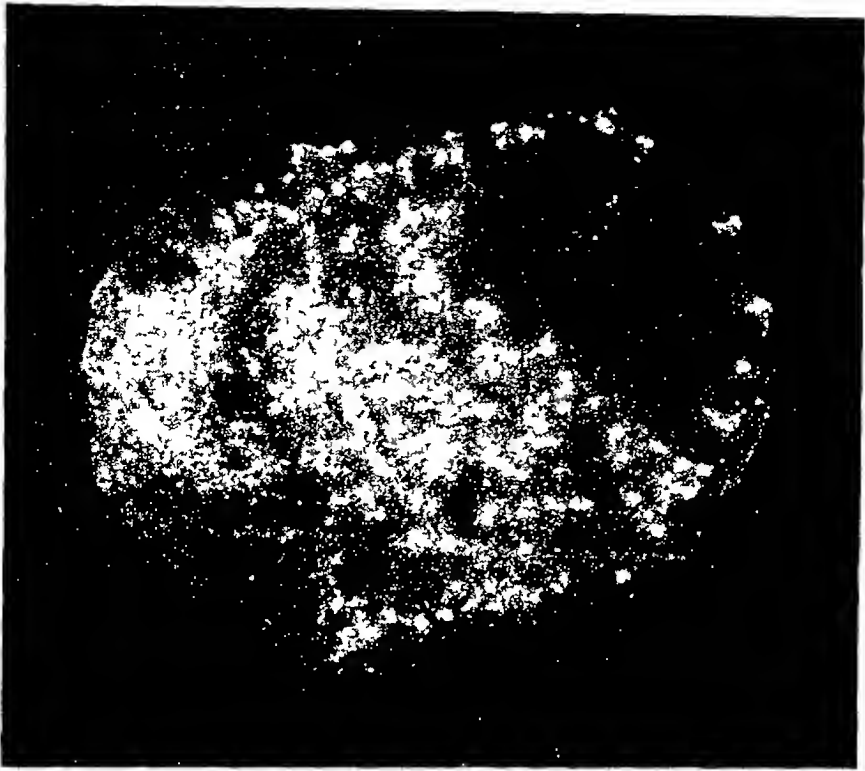


Fig 2—Chorioallantoic membrane of chick embryos forty hours after infection with herpes simplex. Each plaque is produced by one infectious unit ( $\times 30$ )



Fig 3—Section of chorioallantoic membrane infected with herpes simplex. A single plaque is present consisting of a proliferation of epithelial cells. Hematoxylin and eosin ( $\times 80$ )



Fig 4—High power section of Fig. 3. Marked proliferation of epithelial cell many of whose nuclei show margined chromatin and contain a characteristic homogeneous ground glass inclusion body. Hematoxylin and eosin ( $\times 600$ )

ml of the specimen to be tested. The hole was protected from dust by a piece of Scotch tape folded in such a way that the hole in the shell was not tightly sealed. If the hole is sealed completely the true air sac refills and the false air sac is gradually obliterated.

After inoculation the eggs were kept on their sides and incubated for forty eight to seventy two hours at 35 to 36° C. The identity of any virus isolated was established by (a) the gross appearance of the plaques on the chorioallantois (Fig 2), (b) by serial passage on the chorioallantoic membrane (c) by demonstration of the typical intranuclear inclusion bodies in histologic sections of the infected chorioallantoic membrane (Figs 3 and 4) (d) by neutralization tests with the newly isolated virus and hyperimmune rabbit serum.\*

#### APPEARANCE OF INFECTED MEMBRANES

*Gross Characteristics*—The appearance of herpes simplex on the chorioallantoic membrane is well illustrated in Fig 2. Clear cut infection of the membrane with herpes virus produces small white plaques\* on primary isolation, these plaques may be 0.1 to 1.0 mm in diameter, but after several serial passages they average 0.5 to 1.0 millimeter. They are usually round or oval but may be elongated or tailed, they are dense in the center, with only a slight halo of mesodermal reaction. If the plaque is wiped off, which is easily accomplished, the mesodermal reaction can be seen as a faint shadow. If the infected membranes are incubated more than forty eight hours small satellite plaques may appear and they eventually interfere with an accurate enumeration of the original

\*For etymological reasons we prefer the term plaque to that of pock since the lesion consists of superficial proliferation of epithelial cells which appear to be applied to the surface (a plaque) rather than consisting of a pustule (a pock).

plaques In many successful isolations, there was gross evidence of virus on the first passage However, some virus strains grew poorly at first, and nonspecific opacities on the chorioallantoic membrane could be confusing, so that at least three serial passages were made before discarding a specimen as negative

*Microscopic Characteristics*—As soon as typical plaques were evident, a piece of the membrane was fixed for histologic examination Examination of the tissue under low power showed the plaque to be composed of proliferated ectodermal cells, with a minimum of inflammatory reaction in the mesodermal and endodermal cell (Fig 3) layers Many of the ectodermal cells in the plaque contained homogeneous intranuclear inclusion bodies (Fig 4) similar to those seen in the infected rabbit cornea or brain (Fig 1)

Hematoxylin and eosin staining of Zenker's acetic acid fixed tissue has been satisfactory for both animal and egg tissues

#### SUMMARY AND CONCLUSIONS

1 For the isolation and identification of herpes simplex virus, the chorioallantoic membrane of the embryonated egg is as reliable as the rabbit cornea

2 A suitable nonalleergic diluent for preserving the virus is 0.5 per cent gelatin in physiologic saline phosphate buffered to pH 7.2

3 Contaminated materials must be treated with an antibiotic mixture before inoculation A final concentration of penicillin 500 units per milliliter and streptomycin 100  $\mu$ g per milliliter is nontoxic to the herpes simplex virus and to the egg, but is bactericidal for saliva or skin washings For specimens containing yeasts not inhibited by penicillin and streptomycin, the addition of crystal violet in a final concentration of 1 to 50,000 is effective

4 The gross and microscopic appearances of herpes infected chorioallantoic membranes are described

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## THE THERAPEUTIC EFFECT OF TRYPTOPHANE IN HUMAN PELLAGRA

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A METABOLIC relationship between tryptophane and niacin has been demonstrated in laboratory animals and in man. Kiehl, Tepley, Sarna and Elvehjem<sup>1</sup> showed that niacin deficiency induced in rats by a corn diet could be corrected by tryptophane as readily as by niacin. A similar metabolic relationship has been demonstrated in the pig<sup>2</sup> and the horse<sup>3</sup> and the cotton rat.<sup>4</sup> More recently Suttell and Goldsmith<sup>5</sup> and independently Perlzweig, Rosen, Levitas and Robinson<sup>6</sup> have shown that human adults and infants given 1 to 5 Gm of tryptophane daily excrete a much larger amount of N<sup>1</sup> methyl nicotinamide in the urine than is found under basal conditions.

These experiments were suggested by the observation that diets which depend largely on corn and corn products for protein are more highly pellagragenic than diets equally low in niacin but which contain diversified proteins. The mechanism for such an effect was obscure until Kiehl, Tepley, Sarna and Elvehjem<sup>1</sup> showed that rats, a species not affected adversely by a diet deficient only in niacin, developed signs of niacin deficiency when fed a corn diet. Tryptophane restored these animals to health and it was suggested that a deficiency of tryptophane in the corn diet was the controlling factor. At first it seemed likely that tryptophane exerted its protective effect through the synthesis of niacin by enteric microorganisms.<sup>7</sup> More recent evidence, however, indicates that tryptophane may be a direct precursor of niacin, that the conversion occurs after absorption<sup>8-12</sup> and is influenced by pyridoxine.<sup>9-11, 12</sup>

The final proof of the effectiveness of this conversion in man depends upon the demonstration that tryptophane will relieve the acute manifestations of clinical pellagra. We wish to report such observations on one patient with acute pellagrous glossitis, dermatitis, vaginitis and diarrhea and another with chronic pellagrous dermatitis.

Each patient was fed a diet devoid of vitamin C and low in vitamins of the B complex. It had the following composition: calories 3127, protein 41.7 Gm, fat 27 Gm, thiamine 0.42 mg, niacin 4.2 mg, riboflavin 0.60 mg, vitamin C 0 mg, tryptophane 0.07 gram. Tryptophane was administered orally in 2 Gm doses three times daily and twenty-four hour urine specimens were collected whenever possible and analyzed for N<sup>1</sup> methyl nicotinamide.<sup>13</sup> Normal values in our laboratory for N<sup>1</sup> methyl nicotinamide in the urine range from 2.5 to 5.5 mg per twenty-four hours. Clinical observations were made daily by at least two observers.

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## CASE REPORTS

CASE 1—M O, a 30 year old, unemployed, colored woman, was admitted to the Cincinnati General Hospital on July 15, 1948, complaining of painful feet, paresthesias, burning, numbness and swelling of the legs of two months' duration. Diarrhea consisting of four to eight brown liquid stools per day had been present for one month. She also had noted an eruption over the forearms and legs of unknown duration. She had lost 15 pounds of weight.

The patient stated that she ate two meals per day consisting of fatty pork three times a week, six eggs per week, one pint of milk per day, and some vegetables. She was addicted to alcohol. The dietary history could not be verified.

Physical examination revealed a chronically ill, malnourished Negress, moderately well oriented but with poor memory and below normal intelligence. Blood pressure, 140/90, temperature, 99° F, pulse, 90, respirations, 24. There were perfectly symmetrical pellagrous skin lesions on the wrists, dorsal forearms, ankles, legs, and knees. These were deeply pigmented, hyperkeratotic, and sharply demarcated from the surrounding normal skin. Over the bridge of the nose were numerous small hyperkeratotic hair follicles. The tongue was pale with the exception of the tip and edges where it was smooth, swollen, and spotted with clusters of very red, congested papillae, producing fiery red areas on a pale background. There were also spotty areas of stomatitis with one eroded area on the buccal mucous membrane. The eyes, neck, heart, and lungs were normal. The abdomen was normal except for scars from a bullet wound and a laparotomy. The vaginal examination revealed inflamed mucous membrane. There was moderate edema of the ankles. The neurological examination revealed normal reflexes and motor power, but there was marked hyperesthesia of the right foot and moderate hyperesthesia of the left foot from the ankles down, with afterglow in both feet.

**Laboratory Data** On admission, the erythrocytes numbered 3.40 million per cubic millimeter, the hemoglobin, 9.8 Gm per 100 cc, the hematocrit, 32 per cent, the reticulocytes, 3.8 per cent, the platelets, 289,000 per cubic millimeter, mean corpuscular volume, 94 cubic microns, mean corpuscular hemoglobin, 29 micromicrograms, mean corpuscular hemoglobin concentration, 31 per cent, and the white blood cells, 6,200 per cmm with a normal differential count. The bone marrow showed mild hypoplasia of all elements with a slight increase in plasma cells. The blood urea nitrogen was 3.7 mg per 100 cc, the prothrombin concentration, 65 per cent, the Kahn test, negative, thyroxine turbidity, 2 units, thymol flocculation, +, and cephalin flocculation, +++ in twenty four and forty eight hours. Serum

TABLE I. A TABULAR SUMMARY OF THE RESPONSE TO TREATMENT IN CASE 1, A PERSON WITH ACUTE PELLAGRA, PARTICULARLY THE EFFECT OF TRYPTOPHANE ON THE GLOSSITIS, VAGINITIS, DIARRHEA AND TWENTY FOUR HOUR EXCRETIONS OF N<sup>1</sup> METHYLNICOTINAMIDE IN THE URINE

HOSPITAL DAY	1	2	3	4	5	6	7*	8	9	10	11
Tryptophane (Gm per day)			6	6	6	6	4				
Niacin (mg per day)								50	50	50	50
Severity of glossitis and vaginitis	++	+++	+++	++	+	0	0	0	0	0	
Severity of encephalopathy	+	+	+++	+++	++	+++	++→0	0	0	0	
Severity of diarrhea	++	++	++	++	++	++	++→0	0	0	0	
Urinary excretion of N <sup>1</sup> methyl nicotinamide (mg per 24 hr)			0.063			0.720		2.520		4.216	5.040

\*Fifty milligrams of thiamine were administered on this day and 25 mg on each day thereafter.

bilirubin was 0.15 mg. per 100 cc prompt 0.5 mg. per 100 cc total. The liver biopsy showed severe fatty vacuolization. The urine was negative and the stool a liquid brown color without blood, pus cells, ova or parasites. The electrocardiogram was normal. The total protein was 7.56 Gm per 100 cc with albumin 3.3 Gm per 100 cc and globulin 4.2 Gm per 100 cc; these tests were repeated in one week and the total protein was 6.2 Gm per 100 cc, albumin 2.65 Gm per 100 cc and globulin 3.55 Gm per 100 cubic centimeters. The serum vitamin C was 0 mg. per 100 cc serum vitamin A was 40  $\mu$ g. per 100 cc carotene, 28  $\mu$ g. per 100 cubic centimeters. Thiamine excretion in the urine in twenty-four hours was 55  $\mu$ g. and after a test dose of 5 mg. by mouth the patient excreted less than 1 per cent of the test dose in four hours. Riboflavin urinary excretion in twenty-four hours was 434.5  $\mu$ g. and in four hours after 5 mg. of riboflavin by mouth 2.94 milligrams. For urinary N-methylnicotinamide see Table I. X-rays of chest and abdomen were negative.

The patient was placed at bed rest and given a diet low in vitamin B complex and free of vitamin C. Because of the severe diarrhea twenty-four hour urine specimens could not be obtained prior to treatment.

On the second hospital day the tongue was redder and had a small serpiginous ulcer on the right lateral margin. That afternoon the patient was given 50 mg. of Demerol in preparation for a liver biopsy. That night he began to confabulate and had hallucinations. This psychotic behavior was thought to be due to B complex deficiency encephalopathy exaggerated by Demerol. On the morning of the third day tryptophan 2 Gm three times daily was started and in twenty-four hour urine specimen was obtained. On the next day the ulcer on the tongue began to heal although red papillae were still evident. The patient was still confused and paranoid and had hallucinations. By the fifth hospital day the tongue was much improved, the ulcer had healed but a few red papillae remained which disappeared by the next day. The psychotic behavior and the encephalopathy continued, however, and since severe vitamin B complex deficiency particularly of thiamine was thought to be the cause the patient was given 50 mg. thiamine and 10 mg. riboflavin on the seventh hospital day over an eighteen hour period. She was also given 2,000 cc of 5 per cent glucose in water intravenously. There was a rapid response. By that evening the psychotic manifestation had vanished. At this time the acute aspects of the skin lesions were regressing rapidly, the tongue was healed, the diarrhea controlled and the encephalopathy had cleared. Since tryptophan was available in only a limited quantity it was discontinued and a B complex material which contained 100 mg. of niacinamide per cubic centimeter was started. The patient received  $\frac{1}{2}$  cc at this time and on each day thereafter. The peripheral neuritis responded slowly but had cleared almost completely after two more weeks of treatment. The patient was discharged after twenty-six days in the hospital.

CASE 2.—H. J., a 62-year-old colored common laborer was admitted to Cincinnati General Hospital on Sept. 23, 1948, from the city workhouse for study. He had no complaints except for skin lesions of four weeks' duration which he attributed to burns contracted while lying drunk in the sun. He had noticed some numbness and tingling of the feet and mild diarrhea for an undetermined period of time.

His appetite was good. His diet consisted of ham skins, ham fat, potatoes, onions, celery, and cabbage all combined to make stew which he ate one to two times a day. He admitted heavy alcohol intake of at least one quart of Dr. (denatured alcohol) per day. By mistake he had been given one vitamin capsule containing 30 mg. of niacin while in the workhouse hospital one day before admission.

In 1939 he had been in Cincinnati General Hospital with a diagnosis of pellagra, dermatitis and glossitis, polyneuritis and chronic alcoholism. On that admission the skin and mouth lesions cleared after treatment with niacin.

The physical examination revealed a well-developed, poorly nourished Negro man who did not appear ill. There were perfectly symmetrical pellagrous lesions involving the extensor surface of the forearms which were deeply pigmented, hyperkeratotic, scaly, and sharply demarcated from the surrounding normal skin. There were similar lesions

TABLE II THE EFFECT OF TRYPTOPHANE ON THE EXCRETION OF N<sup>1</sup> METHYLNICOTINAMIDE IN THE URINE OF CASE 2, A PERSON WITH CHRONIC PELLAGROUS DERMATITIS, THIS PATIENT RECEIVED 30 MG OF NIACIN INADVERTENTLY BEFORE THIS STUDY WAS BEGUN

DATE	9/23/ 48	9/24/ 48	9/25/ 48	9/26/ 48	9/27/ 48	9/28/ 48	9/29/ 48	9/30/ 48	10/1/ 48	10/2/ 48	10/3/ 48	10/4/ 48	10/5/ 48	10/6/ 48
Tryptophane dosage (Gm / day)					4	6	6	4						
N <sup>1</sup> methyl nicotinamide urinary excretion (mg / 24 hr)		464	111	133	184	294	3936	7665				1785		

involving the collar area of the neck, the ear lobes, and to a lesser extent the groin and feet. The tongue showed only papillary atrophy along the margins. There was severe hyperesthesia and decreased vibration perception in the feet, and the ankle jerks were absent.

**Laboratory Data** The erythrocytes numbered 251 million per cubic millimeter, the hemoglobin, 9.06 Gm per 100 cc, and the white blood cells, 7250 per cubic millimeter with a normal differential count. The urine was normal. The serum protein was 6.85 Gm per 100 cc, urinary thiamine was 17.4 µg in twenty-four hours, and after a test dose (0.5 mg intramuscularly) 4.4 per cent was excreted in four hours. Urinary riboflavin was 1.16 mg in twenty-four hours. See Table II for N<sup>1</sup> methyl nicotinamide excretion.

The patient was placed on a diet low in vitamin B complex and free of vitamin C, and twenty-four hour urine specimens were collected. After three days he was given tryptophane, 2 Gm thrice daily until a total of 20 Gm was given. By the end of the first week the skin lesions had begun to clear and disappeared gradually during the next two weeks. The patient was discharged after twenty-one days in the hospital.

#### DISCUSSION

In the first case tryptophane orally in total daily doses of 6 Gm induced a remission in the acute pellagrous glossitis, stomatitis, vaginitis, and diarrhea. Concomitantly there was an increase in N<sup>1</sup>-methyl nicotinamide in the urine from extremely low to almost normal levels. Subsequently 50 mg nicotinamide daily induced a rapid and great increase in the amount of N<sup>1</sup>-methyl nicotinamide in the urine suggesting that tryptophane may have replenished the body stores of nicotinamide. In this patient the encephalopathy did not clear completely until thiamine was given, which suggests that it was due at least in part to thiamine deficiency.

The second case is included to show that tryptophane increased the amount of N<sup>1</sup>-methyl nicotinamide in the urine of a pellagrum rapidly to high levels just as it will in normal persons on diets restricted in niacin.<sup>4</sup> This subject was not ill when he was admitted to the hospital and it is probable that tissue niacin had been restored by the workhouse diet and the one dose of 30 mg niacin before he was hospitalized. The skin lesions in this patient, though typically pellagrous, were too chronic and indolent to allow critical judgment on the therapeutic effect of tryptophane.

# SUMMARY AND CONCLUSIONS

Tryptophane in 6 Gm oral doses induced a remission in typical acute pellagrous lesions

It caused an increased excretion of N<sup>1</sup> methylnicotinamide in the urine of pellagrins at a slower rate but in the same manner as in normal persons

These observations suggest that tryptophane and niacin are closely related in their role in human as well as in animal nutrition

We wish to acknowledge the technical assistance of Mrs Betty Fichter in performing the N<sup>1</sup> methylnicotinamide determinations and to thank Dr A Gibson of Merck & Co Inc, for a generous supply of tryptophane

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TABLE II THE EFFECT OF TRYPTOPHANE ON THE EXCRETION OF N<sup>1</sup> METHYLNICOTINAMIDE IN THE URINE OF CASE 2, A PERSON WITH CHRONIC PELLAGROUS DERMATITIS, THIS PATIENT RECEIVED 30 MG OF NIACIN INADVERTENTLY BEFORE THIS STUDY WAS BEGUN

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Trypto phine dosage (Gm/ day)					4	6	6	4						
N <sup>1</sup> methyl nicotin amide urinary excretion (mg/ 24 hr)		464	1 11	1 33	3 84	2 94	3 936	7 665				1 785		

involving the collar area of the neck, the ear lobes, and to a lesser extent the groin and feet. The tongue showed only papillary atrophy along the margins. There was severe hyperesthesia and decreased vibration perception in the feet, and the ankle jerks were absent.

*Laboratory Data* The erythrocytes numbered 2.51 million per cubic millimeter, the hemoglobin, 9.06 Gm per 100 cc, and the white blood cells, 7,250 per cubic millimeter with a normal differential count. The urine was normal. The serum protein was 6.85 Gm per 100 cc, urinary thiamine was 17.4 µg in twenty-four hours, and after a test dose (0.5 mg intramuscularly) 4.4 per cent was excreted in four hours. Urinary riboflavin was 1.16 mg in twenty-four hours. See Table II for N<sup>1</sup> methylnicotinamide excretion.

The patient was placed on a diet low in vitamin B complex and free of vitamin C, and twenty-four hour urine specimens were collected. After three days he was given tryptophane, 2 Gm three daily until a total of 20 Gm was given. By the end of the first week the skin lesions had begun to clear and disappeared gradually during the next two weeks. The patient was discharged after twenty-one days in the hospital.

#### DISCUSSION

In the first case tryptophane orally in total daily doses of 6 Gm induced a remission in the acute pellagrous glossitis, stomatitis, vaginitis, and diarrhea. Concomitantly there was an increase in N<sup>1</sup>-methylnicotinamide in the urine from extremely low to almost normal levels. Subsequently 50 mg nicotinamide daily induced a rapid and great increase in the amount of N<sup>1</sup>-methylnicotinamide in the urine suggesting that tryptophane may have replenished the body stores of nicotinamide. In this patient the encephalopathy did not clear completely until thiamine was given, which suggests that it was due at least in part to thiamine deficiency.

The second case is included to show that tryptophane increased the amount of N<sup>1</sup>-methylnicotinamide in the urine of a pellagrin rapidly to high levels just as it will in normal persons on diets restricted in niacin.<sup>4</sup> This subject was not ill when he was admitted to the hospital and it is probable that tissue niacin had been restored by the workhouse diet and the one dose of 30 mg niacin before he was hospitalized. The skin lesions in this patient, though typically pellagrous, were too chronic and indolent to allow critical judgment on the therapeutic effect of tryptophane.

## METHODS AND MATERIALS

The radioiron had an activity of 13.5 mc of Fe 59 and 3.1 mc of Fe 56 per gram at the beginning of the experiments. It was given orally or by stomach tube in the form of ferrous ammonium sulfate with milk or water as the vehicle. Samples of tissues and excreta were aseptically collected and the iron was electroplated by the method of Losburgh, Flexner and Cowie. Radioactivity was measured with a thin mica end window Geiger counter. Tissues for histochemical study were fixed in Carnoy's fluid and formalin or alcohol formalin and were stained for iron by Dry's modification of Perl's method<sup>3</sup> or by Gomori's method<sup>4</sup>. Radioautographs were prepared in duplicate. One paraffin section subsequently stained by Dry's method was used for a routine contact radioautograph with an eco non screen x-ray film. A second section stained for iron was coated with collodion and a liquid photographic emulsion was poured on the slides and allowed to set in the dry. After proper exposure the preparation was developed, fixed, dried, mounted in xylol Christe and covered with a cover slip.

Animals used were guinea pig, albino rats and one dog. Guinea pigs of 240 to 450 grams were maintained for at least two weeks on a stock pellet diet containing .02 mg of iron per 100 grams. All food and water were withheld for twenty four hours before giving 20 mg of radioiron by mouth.

A number of guinea pigs made anemic one week prior to the experiment by one bleed mg of 5 to 10 cc from the heart also were given radioiron. Control animals both normal and anemic were treated identically except that no radioiron was administered to them. Animals were killed thirty minute to twenty two hours after administration of the test dose of iron. Controls were killed at identical intervals during the same period. The net absorption of the single dose of tagged iron was calculated by subtracting from the total dose that iron recovered in the excreta and gut content. Portions of mesenteric and cervical nodes, spleen and liver and samples of blood were taken and the content of radioiron was determined as micrograms per gram of tissue or blood.

Weanling rats of 40 to 60 grams were kept on purified diets containing 61 per cent sucrose, 25 per cent casein, 10 per cent Crisco and 4 per cent of Hubbel's salt mixture from which the iron was omitted. To each kilogram of diet were added 20 milligrams nicotin, 40 milligrams calcium pantothenate, 0.01 milligram biotin, 20 milligrams riboflavin, 10 milligrams thiamine hydrochloride, 10 milligrams of pyridoxine, 1 gram of choline and 4 milligrams vitamin K. All rats received 3 milligram alpha tocopherol in 0.05 cc ethyl laurate once a week, and 2,000 units of vitamin A and 400 units of vitamin D in corn oil twice a week. The basic diet contained 15 mg of iron per 100 Gm of diet of which 1 to 2 mg were iron impurities in the salt mixture and the remainder was in the purified calcium. Forty eight rats used in one experiment were divided into three equal groups. Group A received a high iron diet, 50 mg of iron as ferric phosphate being added to each 100 Gm of diet. Groups B and C received the basic low iron diet without iron supplement. The animals were kept on these diets for five weeks prior to the administration of radioiron. During the fifth week Group C animals were bled 2 per cent of their body weight on alternate days until the hemoglobin values were 6 to 9 Gm per cent (14 to 17 Gm per cent in Groups A and B). At the end of the fifth week all animals were starved for twenty four hours after which 1 mg of radioiron was given in water by stomach tube; the control animals received nothing.

In another experiment designed to eliminate the possible effects of twenty four hours of starvation sixteen rats were kept for four weeks on a high iron diet. Three days prior to the administration of 1 mg of radioactive iron as ferrous ammonium sulfate half the animals (A) were placed on a low iron diet for the remainder of the experiment while the other animals were kept on a high iron diet throughout (A). Food was not withheld even after the test meal was given. Animals were killed two, six and twenty four hours after the administration of the test dose. Control animals on the same diets were given no test dose of radioiron but were killed at corresponding times.

In order to determine absorption of iron by way of the lymphatics a 10 kilogram female ferrier was prepared by inserting in the thoracic duct a plastic cannula which was carried through the skin incision. After two days the dog was given 57 mg of tagged iron

TABLE I COMPARISON OF VISIBLE IRON AND RADIOIRON IN THE DUODENUM OF GUINEA PIGS AT VARIOUS INTERVALS AFTER A TEST MEAL OF 20 MG OF RADIOIRON AS FERROUS AMMONIUM SULFATE IN 6 PER CENT SUCROSE SOLUTION

EXPERIMENTAL CONDITIONS	GUINEA PIG	TIME FROM TEST MEAL TO DEATH (HR)	VISIBLE IRON			RADIOIRON	
			EPITHELIAL GRANULES	BASAL EPITHELIAL FLUSH	IN PHAGOCYTES	RADIOAUTOGRAPHIC DENSITY	DISTRIBUTION
Normal guinea pigs given test meal by mouth	26	1	±	+	++	++++	Epithelium
	27	2	++	±	++	++++	Epithelium
	28	2	++++	++	+++	++++	Epithelium
	29	4	+	±	±	+++	Epithelium
	30	4	+++	++	++	+++	Epithelium
	31	8	++++	++	+++	++	Epithelium
	32	8	+++	±	+++	++	Epithelium
	33	24	+	±	+++	++	Epithelium
	34	24	++++	±	+++	++	Epithelium
	35	48	±	++	+++	++	Epith & phag
	36	48	+	±	+	++	Epith & phag
	37	72	+++	-	++++	++	Epith & phag
	38	72	+	-	++	++	Epith & phag
Normal control guinea pigs given no test meal	39	40*	±	±	+++	No radioiron present	
	40	40*	+++	-	++		
	41	66*	+++	-	+++		
	42	90*	+++	-	++		
Anemic guinea pigs given test meal by mouth	44	1 1/2	++	+	++	++++	Epithelium
	45	1 1/2	+	+	+++	++++	Epithelium
	46	2	±	-	++	++++	Epithelium
	47	2	+++	±	++++	+	Epithelium
	48	24	+	-	+	++	Epith & phag
	49	24	+++	±	+++	++	Epith & phag
	50	24	++	-	±	++	Epith & phag
	51	24	+++	±	++	++	Epith & phag
Anemic control guinea pigs given no test meal	52	40*	+	±	+	No radioiron present	
	53	40*	+++	-	++		
	54	66*	+	±	++		
	55	66*	+	±	+		

\*Time elapsed since last food was taken

as ferrous ammonium sulfate by stomach tube in 300 cc of whole milk. The entire flow of thoracic duct lymph for the following six hours was collected in three parts. Blood samples were collected at three and five hours.

## RESULTS

Histochemically demonstrable iron will be referred to as visible iron. Radioactive iron demonstrated either by radioautographs or by Geiger counters will be referred to as radioiron. Control animals will be those handled exactly as their experimental counterparts except that they received no test dose.

In Tables I to III are tabulated the results of the experiments with guinea pigs. Inspection of these tables reveals that we were unable to confirm some of the observations of Gillman and Ivy regarding visible iron in guinea pigs. The distribution and amount of visible iron in the various organs evidently did not

TABLE II COMPARISON OF VISIBLE IRON AND RADIOIRON IN THE DUODENUM OF GUINEA PIGS AT VARIOUS INTERVALS AFTER A TEST MEAL OF RADIOIRON IN SUCROSE MILK AND WATER VEHICLE

EXPERIMENTAL CONDITIONS	GUINEA PIG	TIME FROM TEST MEAL TO DEATH (HR)	VISIBLE IRON			RADIOIRON	
			EITHER LIAL OR ANNULES	BASAL EPITHELIAL PLUSH	IN PHAGOCYTES	RADIOAUTOGRAPHIC DENSITY	DISTRIBUTION
Normal guinea pigs given test meal by mouth	56	2	+	+	++	+	Epithelium
	57	2	++	++	++	++	Epithelium
	58	6	+	+	++	+	Epithelium
	59	6	++	+	++	+	Epithelium
	60	24	++	+	++	+	Epith and phag
	61	24	±	-	+	++	Epith and phag
Normal control guinea pigs given no test meal	62	20*	+	-	+++	No radioiron present	
	63	40*	+++	+	+		
Anemic guinea pigs given test meal by mouth	64	2	++	+	±	+	Epithelium
	65	2	+++	+	+	+	Epithelium
	66	6	++++	+	+++	+	Epithelium
	68	24	++	-	+++	++	Epith and phag
	69	24	+++	+	++	++	Epith and phag
Anemic control guinea pigs given no test meal	70	20*	+++	+	±	No radioiron present	
	71	40	±	-	+		

Time elapsed since last food was taken

change regularly in a cyclic form after a single iron meal under the present experimental conditions. In fact in view of the erratic variation in the experimental groups and the occurrence of the same range of variation in the control groups which had received no test meal of iron one would be led to doubt whether the test meal had any particular effect on the visible iron and would be inclined to believe that the visible iron must not have originated in the test meal.

The radioautographs and other tracer studies on these same animals indicate clearly that most of the visible iron did not originate in the test meal. There is no correlation between the distribution or amount of visible iron and the distribution or amount of radioiron. For example in the duodenal epithelium of a given animal the visible iron varied erratically from one villus to another, while the radioiron as shown by radioautographs, diminished smoothly from a maximum near the pylorus to a minimum in the proximal jejunum.

Similarly we were unable to confirm the observation that the formation of epithelial granulation iron was reduced in anemic guinea pigs. In fact in Table II one notes that anemic animals tended to have more granules than normal animals. These findings throw some doubt on the suggestion of Gillman and Iv



TABLE III COMPARISON OF VISIBLE IRON AND RADIOIRON IN VARIOUS ORGANS OF NORMAL GUINEA PIGS AFTER TEST MEALS OF RADIOIRON AS DIFFERENT AMMONIUM SULFATE IN 6 PER CENT SUCROSE SOLUTION

EXPERIMENTAL GROUP	GUINEA PIG	TIME FROM TEST MEAL TO DEATH (HR.)	MESENTERIC LYMPH NODE		CERVICAL LYMPH NODE		LIVER		SPLEEN	
			VISIBLE IRON	RADIOIRON (μg/GM.)	VISIBLE IRON	RADIOIRON (μg/GM.)	VISIBLE IRON	RADIOIRON (μg/GM.)	VISIBLE IRON	RADIOIRON (μg/GM.)
Normal guinea pigs given test meal of 20 mg radioiron as ferrous ammonium sulfate in 6% sucrose solution	25	1	++	Not done	±	3.20	-	6.96	±	8.60
	26	1	++	2.86	++	0.41	-	11.8	+	0.53
	27	2	+	4.00	++	6.71	-	Not done	+++	10.87
	28	2	+++	0.47	-	0.78	-	9.15	++	3.37
	29	4	++	0.67	++	21.94	-	13.68	++	0.75
	30	4	++++	0.77	-	1.03	-	6.61	±	0.95
	31	8	++++	3.04	-	1.10	-	6.17	+++	0.59
	32	8	++++	1.84	++	4.40	-	6.39	+	0.69
	33	24	++	2.23	-	0	±	15.33	++	1.44
	34	24	++++	2.17	±	0	±	10.98	++++	3.25
	35	48	++++	4.60	-	0.66	±	12.94	++++	7.54
	36	48	±	Not done	-	1.05	±	13.22	+	4.95
	37	72	++++	2.70	-	1.26	±	9.62	++++	4.87
	38	72	-	3.54	-	1.09	±	Not done	+++	4.02
Normal control guinea pigs given no test meal	39	40*	+++	-	+	-	-	-	++++	-
	40	40*	++	-	-	-	-	-	+++	-
	41	66*	-	-	-	-	-	-	++++	-
	42	90*	++	-	-	-	-	-	++	-

\*Time elapsed since last food was taken

TABLE IV VISIBLE IRON IN THE DUODENUM AND SPLEEN OF RATS FED DIFFERENT DIETS

GROUPS	VISIBLE IRON IN DUODENUM				VISIBLE IRON IN SPLEEN
	DUODENUM		SPLEEN		
	AFTER TEST DOSE OF 1 MG IRON	CONTROLS NO TEST DOSE	AFTER TEST DOSE OF 1 MG IRON	CONTROLS NO TEST DOSE	
A—High iron diet	Present in 8 Absent in 1	Present in Absent in	Present in 9 Present in 7	Present in 4 Absent in 2	3+
A—High iron diet plus 7 days low iron diet	Present in 1 Absent in 2	Absent in 4	Present in 7	Present in 4	2+
A—High iron diet	Present in 4 Absent in 1	Absent in 1 Present in 1	Present in 5	Present in 4	2+
B—Low iron diet	Present in 7 Absent in 5	Absent in 1	Absent in 10	Absent in 1	1+ or 2+
C—Low iron diet and bled from tail vein	Present in 6 Absent in 4	Absent in 1	Absent in 8 Present in 2	Absent in 4	-

Groups A, B, and C starved for twenty-four hours prior to administration of test dose.  
Groups A and V not starved.

that the epithelial granular iron is a morphologic expression of mucosal block. The tracer studies were interesting in this connection. Despite the wide variation in amount of visible granular iron in the duodenal epithelium the normal animals all absorbed about 15 per cent of the test meal iron while all anemic animals absorbed about 50 per cent.

The results of the guinea pig experiments were somewhat equivocal as to the role of the reticuloendothelial and lymphatic systems. The amount of visible iron in the lymph nodes, spleen, and liver did not correlate with the amount of radionon. However, since most of the samples of these tissues were obtained more than two hours after the test meal and since absorption studies indicated that most of the absorption occurred in the first two hours, one could not exclude the possibility that the non might already have entered the blood via the intestinal lymphatics and thoracic duct.

After efforts at quantitative collection of thoracic duct lymph in guinea pigs, rats, and rabbits had failed, the dog experiment was done for the purpose of determining to what extent iron entered the organism via the thoracic duct. The entire flow of thoracic duct lymph for six hours after the radionon test meal contained only 10.28  $\mu\text{g}$  of the test dose of iron. Assuming a blood volume equivalent to 8 per cent of body weight, the blood samples collected at three and five hours indicated that the blood stream alone contained 104 and 196  $\mu\text{g}$  of the radionon respectively. In this one dog, absorbed non was transported almost exclusively via the portal vein.

Having failed to confirm these findings of Callman and Ivy and having arrived at no clear understanding of the significance of visible non, we undertook the rat studies in the hope that careful control of dietary non over relatively long periods might yield more illuminating results.

The results are tabulated briefly in Table IV. Visible non occurred in much smaller amounts in rats than in guinea pigs, although when present it occurred in the same form and same locations. There was no clear cut post prandial cycle and no correlation between visible non and radionon. In a series

of unpublished experiments we were unable to relate the variation in amount and distribution of visible iron to the type of iron in the diet and, although a variety of ferrous and ferric compounds were tested as well as the stock guinea pig diet, we have been unable to increase the visible duodenal iron in rats to the levels seen in guinea pigs

In rats, as in guinea pigs, the amount of visible iron varied considerably even in groups having been maintained for weeks on the same carefully controlled iron intake. However, in twenty-six of forty-seven rats receiving high iron diet or test meal of iron within twenty-four hours before being killed, visible iron was demonstrated in the duodenal epithelium while it was absent in all of nine rats ( $A_1$  controls, B controls, and C controls) which had received a low iron diet for at least three days and no test meal. This suggests that considerable iron has been present in the diet within the last few days of life when visible iron is found in the duodenal epithelium. In twenty-nine of thirty-one rats given a high iron diet for several weeks, visible iron was present in the phagocytes of the duodenal stroma but was absent in twenty-three of twenty-five rats given a low iron diet throughout. Administration of a low iron diet for three days following several weeks of high iron diet (Group  $A_1$ ) did not result in the disappearance of the visible iron in the stroma. Apparently this stromal iron accumulates during periods of considerable iron intake and disappears more slowly than the epithelial iron during periods of low iron intake.

The amount of visible splenic iron in rats showed no relationship to the single test meal but was uniformly high in all rats on a high iron diet, moderate in rats on a low iron diet, and absent in anemic rats on a low iron diet. These findings are consistent with the widely accepted view that visible iron in the spleen is storage iron which reflects general levels of storage iron except in conditions characterized by excessive destruction of red cells.

#### SUMMARY

1 Combined histochemical, radioautographic, and tracer methods were used to study the absorption and distribution of single test meals of radioiron in guinea pigs, rats, and one dog.

2 The iron demonstrated histochemically in the duodenal mucosa, the mesenteric and cervical lymph nodes, liver, and spleen was derived largely from sources other than the single test meal. This visible iron did not undergo well-defined cyclic changes after a single iron meal. It accumulated over a period of days or weeks of continued intake of a diet containing considerable iron. It behaved more like storage iron than iron in transport.

3 The visible granular iron in the duodenal epithelium exerted no demonstrable effect on the amount of iron absorbed, and it did not appear to be a morphologic expression of mucosal block.

4 Most of the radioiron of the test meal traversed the duodenal epithelium rapidly. In one dog it was transported from the intestine via the portal vein—only insignificant amounts being found in thoracic duct lymph.

5 There was no evidence to indicate that the reticuloendothelial system participated directly in the absorption of iron from the intestine or transport of the absorbed iron from the intestine to the liver, blood, and other organs and tissues

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## LABORATORY METHODS

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### NEEDLE BIOPSY OF THE LIVER USING OXIDIZED CELLULOSE AND THROMBIN TO PREVENT HEMORRHAGE

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**D**URING the past fifty years needle biopsy of the liver has been performed for purposes of diagnosis and study of the etiology, pathogenesis, and altered physiology in various diseases of the liver. This relatively simple bedside procedure has been at times a helpful tool for the investigator in attempting to evaluate different regimes of therapy and various liver function tests. Needle biopsy offers several advantages over other methods of obtaining material for microscopic study. A needle biopsy sample of liver tissue can be immediately fixed, stained, and studied without the necessity of attempting to evaluate the autolytic changes that may be encountered at times in post-mortem material. Another advantage of needle biopsy is that repeated studies of the same patient's liver can be carried out at varied intervals, whereas it is not usually feasible to repeat laparotomies merely to examine microscopically sections of the liver. In some instances patients critically ill with cancer have been spared the discomforts of an unnecessary exploratory laparotomy because it has been possible to confirm the presence of hepatic metastasis by means of microscopic examination of material from a diagnostic needle biopsy of the liver. Since this procedure at times offers so much from a diagnostic and clinical standpoint, one might wonder why there have been only about 2,000 instances of its use reported in the literature during the past half century. Examination of the mortality and complications following the use of this procedure as reported<sup>1-11</sup> readily reveals the reason for the lack of a more general interest and application of needle biopsy of the liver. It is noteworthy that eleven of fourteen deaths reported in the literature as resulting from needle biopsy were due to hemorrhage and three were from peritonitis.

#### METHOD

The method which we used in our first 140 biopsies, 119 of which have been reported,<sup>12</sup> has been used extensively in this country and described in previous reports.<sup>4, 7, 9, 12</sup> The Vim Silverman needle was employed through an anterior subcostal approach. The procedure which we are now using and have employed during our last eighty-five biopsies involves the use of the Vim Silverman needle in conjunction with a trocar, plunger, and cartridge for oxidized cellulose\* (see Fig. 1).

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\*The liver biopsy instruments used in this study were made for us by Mr. Harold Breeding, Instrument Technician, Department of Physiology, University of Louisville School of Medicine, and may be procured from Mr. Breeding.

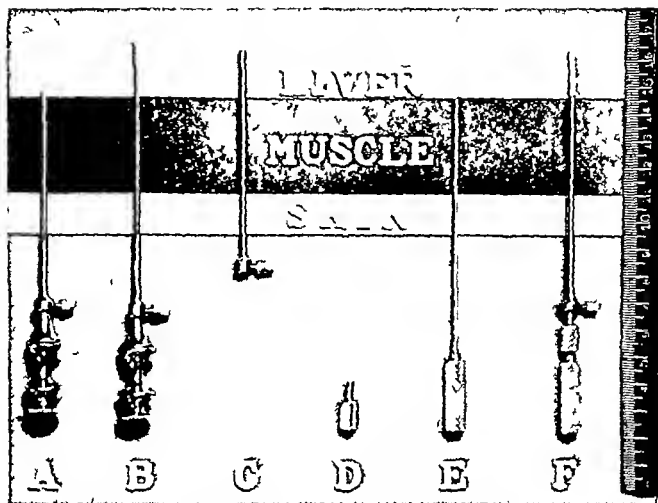


Fig 1—Needle biopsy technique with modified Vim Silverman needle trochar cartridge for oxidized cellulose and plunger (See Method)

Bleeding clotting and prothrombin times are determined prior to biopsy and the patient is given intramuscular sodium phenobarbital sedation. With the patient lying on his back the abdomen is prepared carefully with Merthiolate and alcohol and draped with sterile towels. The skin of the abdominal wall down to and including the peritoneum is then carefully infiltrated with novocain. A 2 to 3 mm incision is made in the skin with a No 11 Bard Parker blade to facilitate the introduction of the trocar and needle. For an enlarged liver the site usually selected is about 2 cm to the right of the midline and just below the costal margin but this varies with the individual case and depends upon the location of palpable nodules or special areas that one might desire to biopsy. The Vim Silverman needle locked inside the trocar by a set screw (as shown in *A* Fig 1) is introduced through the skin and down into the liver until the entire bevel of the Silverman needle is beneath the liver capsule. The stylet is removed and the cutting blades are introduced through the Silverman needle 17 cm into the liver substance (as shown in *B* Fig 1). The cutting blades are then held firmly in place while the trocar and Silverman needle are also pushed down over the cutting blades 17 cm into the liver tissue thus shearing off the tissue protruding from the sides of the cutting blades. The set screw on the trocar is next released and the Silverman needle and cutting blades containing the biopsy are rotated through 360 degrees and removed together from the trocar. At this point the tip of the trocar is still 17 cm down into the liver substance at the base of the biopsy site (as shown in *C* Fig 1). The cartridge (shown in *D* Fig 1) containing oxidized cellulose (Oxycel or Gelfoam) which has been made pliable by soaking in thrombin solution is placed into the trocar and the plunger (shown in *E* Fig 1) is pulled down through the cartridge and trocar forcing the cellulose plug into the base of the biopsy tract in the liver. As this is being done the trocar is withdrawn from the liver leaving the biopsy site packed with a plug of oxidized cellulose and thrombin (as shown in *F* Fig 1). As a further adjunct to the control of bleeding a small piece of gauze is placed over the skin incision and firm pressure is applied directly over the biopsy site with the gloved thumb for approximately five minutes.

The biopsy tissue is removed from the cutting blades and is immediately put into the fixative, or, if glycogen determinations are to be made, is immediately immersed in potassium hydroxide in a weighed container. The patient is kept as flat as possible for six to eight hours after the biopsy and is allowed out of bed in eighteen to twenty four hours if there are no complications apparent or other contraindications for his being ambulatory.

### RESULTS

In our first 119 biopsies without the triocal or oxidized cellulose and thrombin, we had the following complications. Symptoms of shock in three instances (mild and readily overcome, plasma being used in only one case), mild bile peritonitis in two instances. In our present series of eighty-five cases using oxidized cellulose and thrombin, we have had no untoward symptoms or signs of any complication.

### DISCUSSION

The liver biopsy method outlined in this report was developed for the primary purpose of reducing the danger of hemorrhage which has heretofore been the chief cause of mortality associated with this procedure. Proper selection of cases is still necessary, and care in directing the needle is also very important, because perforation of a viscus with resultant peritonitis may occur. Furthermore, it is felt that this procedure will certainly not prevent arterial hemorrhage as in the case reported by Hoffbauer<sup>7</sup>, consequently, care must still be used in observing the patients after this procedure.

### SUMMARY

A method of employing oxidized cellulose and thrombin which may aid in preventing fatal hemorrhage from the liver following needle biopsy is herein presented.

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## DETECTION OF BROMATE IN BLOOD AND URINE

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WITH the widely prevalent use of bromate solutions in conjunction with hair waving kits of various manufacturers a need has arisen for a rapid qualitative test for bromate ion in blood and urine to establish with surety the presence of bromate in those rare instances in which accidental ingestion is suspected so that appropriate therapeutic measures can be instituted. A test is presented which has high sensitivity and utilizes reagents readily available in any hospital laboratory. Such cases of poisoning are rare, but the frequency of cases in which bromate poisoning is suspected has increased recently. This test will prove of value in making a positive diagnosis.

One of the early uses of an azo dye such as methyl orange in a qualitative analysis for a halate was made by Besemann<sup>1</sup>. Korenman<sup>2</sup> found that the decolorization of methyl orange by bromate ion proceeded much more rapidly than by chlorate, iodate and other oxidizing anions and based a qualitative test for bromate on this decolorization by an acidified solution containing bromate ion. He claimed a sensitivity of 14 gammas in 2 cc of solution. Sarudi<sup>3</sup> evolved a volumetric determination of bromates using methyl orange as an indicator. A comparison of volumetric analyses for bromate using the iodometric technique and using methyl orange as the indicator was made by von Stetina<sup>4</sup> who found that methods using methyl orange as an indicator were in good agreement with the iodometric technique.

Korenman also established the quantities of other substances which obscure the decolorization of dilute methyl orange by 20 gammas of potassium bromate in weak acid solution. A summary of his figures is given in Table I.

As a part of the development of the following procedure, a careful evaluation of some of the commonly available azo dyes was made. Their hues and intensities and relative sensitivities to bromate were studied. See Table II. Methyl orange in the dilution used and in acid medium has a pale pink color which is difficult to see in urine and impossible to use with plasma from blood in which the slightest hemolysis has occurred. Bismark brown has a weak yellow brown color in dilute solution and hence is useless when determining bromate in urine. Congo red and tripan blue are more satisfactory but neither gives as strong a color in the dilution used as does Evans blue. Of the dyes investigated Evans blue was superior in hue and intensity and was equal to any of the others in sensitivity to bromate. A very sharp clean cut test could be obtained with concentrations of potassium bromate of 10 gammas per cubic centimeter of tested fluid. Of all the substances having any likelihood of being

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TABLE I

(By its rapidity in decolorizing 2 drops of 0.015 per cent methyl orange in weak acid, 20 gammas of potassium bromate can be detected in the presence of the indicated amounts of the substances listed below. Of the substances tested, nitrite is shown to be the most likely to interfere. Korenman<sup>2</sup>)

KIO <sub>3</sub>	40 mg	(KBrO <sub>3</sub> KIO <sub>3</sub> = 1 2,000)
KClO <sub>3</sub>	20 mg	(KBrO <sub>3</sub> KClO <sub>3</sub> = 1 1,000)
KNO <sub>3</sub>	50 mg	(KBrO <sub>3</sub> KNO <sub>3</sub> = 1 2,500)
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	3 mg	(KBrO <sub>3</sub> K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> = 1 150)
K <sub>3</sub> Fe(CN) <sub>6</sub>	40 mg	(KBrO <sub>3</sub> K <sub>3</sub> Fe(CN) <sub>6</sub> = 1 2,000)
NaNO <sub>2</sub>	300 γ	(KBrO <sub>3</sub> NaNO <sub>2</sub> = 1 15)
NaCl	400 mg	(KBrO <sub>3</sub> NaCl = 1 20,000)
KBr	200 mg	(KBrO <sub>3</sub> KBr = 1 10,000)
(NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	200 mg	(KBrO <sub>3</sub> (NH <sub>4</sub> ) <sub>2</sub> S <sub>2</sub> O <sub>8</sub> = 1 10,000)

present in the blood or urine, only nitrite interfered by shifting the color of the dye from blue to violet or, in higher concentrations, by decolorizing it. This was to be expected since Korenman's figures show that nitrite ion, among the substances he tested, approached nearest to bromate in its power of decolorizing

TABLE II

IN 2 CC SOLUTION	EVANS BLUE 0.01% (0.1 cc)	TRYPAN BLUE 0.01% (0.1 cc)	CONGO RED 0.01% (0.1 cc)	METHYL ORANGE 0.01% (0.1 cc)	BISMARCK BROWN 0.01% (0.1 cc)
KClO <sub>3</sub> 1 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KClO <sub>3</sub> 10 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KClO <sub>3</sub> 100 mg %	Violet	Pale blue— colorless in one hour	Pale blue— colorless in 25 min	Pale pink— colorless in 10 min	Pale yellow
KBrO <sub>3</sub> 1 mg %	Decolorized instantly	Decolorized in stantly	Decolorized in stantly	Decolorized in stantly	Pale yellow
KBrO <sub>3</sub> 10 mg %	Decolorized instantly	Decolorized in stantly	Decolorized in stantly	Decolorized in stantly	Decolorized in stantly
KBrO <sub>3</sub> 100 mg %	Decolorized instantly	Decolorized in stantly	Decolorized in stantly	Decolorized in stantly	Decolorized in stantly
KIO <sub>3</sub> 1 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KIO <sub>3</sub> 10 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KIO <sub>3</sub> 100 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KNO <sub>3</sub> 1 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KNO <sub>3</sub> 10 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
KNO <sub>3</sub> 100 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
Na <sub>2</sub> AsO <sub>4</sub> 1 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
Na <sub>2</sub> AsO <sub>4</sub> 10 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
Na <sub>2</sub> AsO <sub>4</sub> 100 mg %	Blue	Pale blue violet	Pale blue	Pink	Yellow brown
NaNO <sub>2</sub> 1 mg %	Very faint blue violet	Faint blue	Decolorized in stantly	Pink	Pale yellow
NaNO <sub>2</sub> 10 mg %	Decolorized instantly	Faint blue	Decolorized in stantly	Pink, fades rapidly	Pale yellow
NaNO <sub>2</sub> 100 mg %	Decolorized instantly	Very faint blue	Decolorized in stantly	Decolorized instantly	Yellow brown

methyl orange The well known reaction between nitrous acid and primary amines can be utilized to destroy nitrite, simply by the addition of an excess of urea to the acidified solution to be tested When very minute quantities of bromate are present, the addition of urea slows the decolorization of the dye but does not interfere with the test

#### PROCEDURE

To 2 cc of blood plasma or urine to be tested add 1 cc of 3 or 4N HCl If plasma is used, centrifuge after adding the acid to throw down the proteins Add 0.1 cc or 2 drops of 0.01 per cent Evans blue in water solution Immediate fading of the blue color shows the presence of bromate If nitrite is suspected add 0.25 Gm of urea and wait for the evolution of nitrogen to cease before adding the dye When bromate is present in amounts corresponding to 1 mg per cent  $\text{KBrO}_3$  the time required to decolorize the dye in the presence of urea is lengthened to ten or fifteen seconds

#### SUMMARY

A simple and rapid method for the detection and confirmation of the presence of bromate in blood or urine has been described The method is based on the selective decolorization of an azo dye Evans blue by bromate ion

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# THE USE OF AZOALBUMIN AS A SUBSTRATE IN THE COLORIMETRIC DETERMINATION OF PEPTIC AND TRYPTIC ACTIVITY

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THE coupling of a protein with a diazotized aryl amine will produce a chromophoric derivative. Such an azoprotein may be precipitated from solution by trichloroacetic acid to yield a colorless filtrate. If the azoprotein solution is subjected to proteolytic digestion, colored reaction products are formed which are soluble in trichloroacetic acid solution. The intensity of color in the trichloroacetic acid filtrate of the digested substrate is a function of the proteolytic activity of the enzyme solution. These findings have served as a basis for a colorimetric method for the determination of the proteolytic activity of duodenal juice.<sup>1</sup> Sulfanilamide-azocasein, the substrate used, being soluble only in alkaline solution, could not be used for the determination of peptic activity. The present publication reports the preparation of an azoalbumin which is soluble in both alkaline and acid solution and therefore permits the colorimetric method to be adapted to the analysis of gastric as well as duodenal juice.

## EXPERIMENTAL

*Preparation of Sulfanilic Acid Azoalbumin* Solution A: 50 Gm of bovine plasma albumin\* are dissolved in 1 l H<sub>2</sub>O. Ten grams NaHCO<sub>3</sub> suspended in 100 ml H<sub>2</sub>O are added with stirring. Solution B: 0.025 mole sulfanilic acid is dissolved in 200 ml H<sub>2</sub>O containing 0.025 mole NaOH (50 ml of 5.0N), 0.025 mole NaNO<sub>2</sub> added. Solution stirred and 0.05 mole (100 ml of 5.0N) HCl added. Solution stirred two minutes and 0.05 mole NaOH (100 ml 5.0N) added to stirred solution. Solution stirred for five seconds and added at once to Solution A with vigorous stirring.

Material stirred five minutes and dialyzed against cold tap water overnight. The dialyzed solution is then lyophilized. The azoalbumin is a red-orange compound with an absorption maximum at 440 to 450 mμ (Fig. 1). The color-concentration relationship of azoalbumin follows Beer's law (Fig. 2). This is fortunate in that in the calculation of enzyme activity, optical density values may be substituted for substrate concentrations. The azoalbumin is soluble in the pH range below 3 and above 5.

*Digestive Juice* The gastric or duodenal juice sample is centrifuged at about 1,500 rpm for ten minutes and the sample is taken from the relatively homogeneous middle layer. Duodenal juice samples are diluted 1:100 with 0.5N ammonium hydroxide ammonium chloride buffer of pH 9.5. Gastric juice samples are diluted 1:20 with 0.2N hydrochloric acid sodium citrate buffer of pH 1.6.

From the Nutritional Service of the Department of Pediatrics and the Gastrointestinal Section of the Medical Clinic of the University of Pennsylvania Medical School and the Wyeth Institute of Applied Biochemistry.

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\*The preparation used was bovine plasma albumin Fraction V, Armour Company, Chicago.  
†Other albumins may be used.

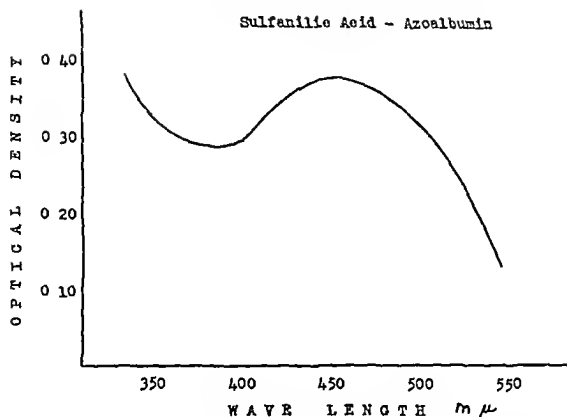


Fig 1—Absorption curve of sulfanilic acid azoalbumin (0.12 mg per milliliter)

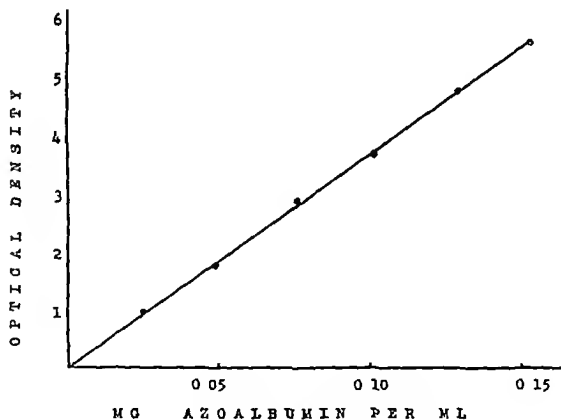


Fig 2—Color concentration relationship of azoalbumin

**Substrate Solution** The azoalbumin is dissolved in the buffer appropriate to the determination at a concentration of 25 mg per milliliter

**Procedure** The details of the assay procedure have been reported in an earlier publication.<sup>1</sup> Each determination is set up in duplicate. One milliliter of substrate solution is pipetted into a test tube, 1 ml of the diluted enzyme sample is added and the mixture digested for thirty minutes at 38° C. The digestion is stopped and undigested substrate removed from solution by the addition of 8 ml of 5 per cent trichloroacetic acid.

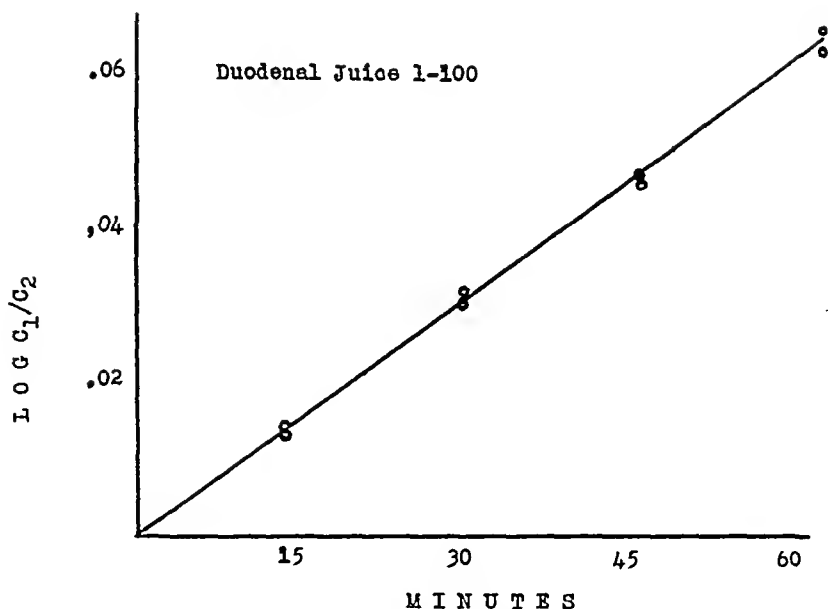


Fig 3—Relationship of proteolytic activity of duodenal juice to time of digestion

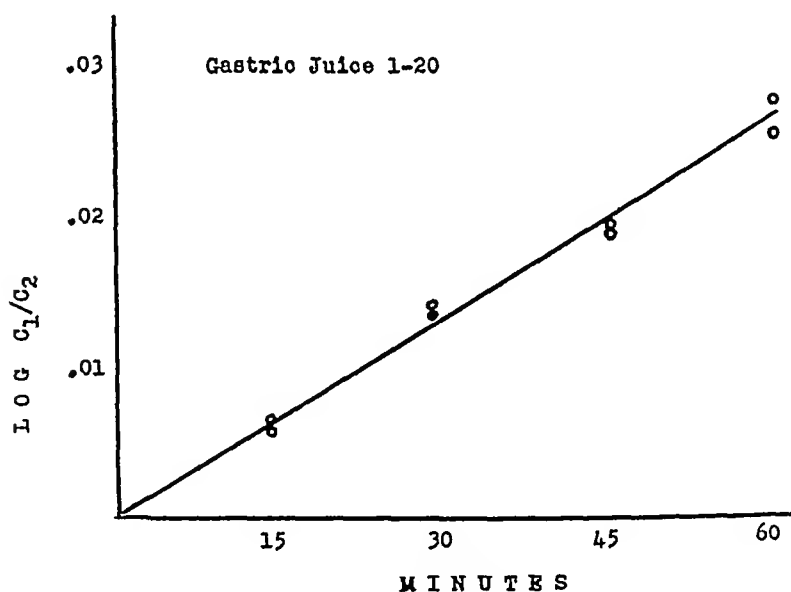


Fig 4—Relationship of proteolytic activity of gastric juice to time of digestion

The contents of each tube are filtered through paper. To a 5 ml aliquot of the filtrate are added 5 ml of approximately 0.5N NaOH and color intensity is read on a photoelectric colorimeter (440 mμ). A substrate blank is prepared by adding 1 ml of buffer to 1 ml of substrate solution and treating the tube in the same manner as the samples. No blank need be run for the enzyme solution since the final dilution in the case of duodenal juice is 1:2,000, and 1:400 for gastric juice.

The activity of the juice is expressed in terms of the velocity constant of the enzymatic reaction,  $K = \frac{1}{t} 2.3 \log \frac{C_1}{C}$ , where  $C_1$  and  $C$  are initial and final protein concentrations respectively after  $t$  minutes of digestion.

Since the color concentration relationship of azoalbumin and its digestion products obeys Beer's law, optical density values may be substituted for  $C$  values. The initial concentration,  $C_1$ , is determined by adding 5 ml of 0.5N NaOH to a 5 ml aliquot of a 1:200 dilution of the substrate solution and then reading the color of the final solution. This optical density value is multiplied by twenty to yield the  $C$  value, since the undigested sample is diluted twenty times more than the filtrate samples. The  $C_2$  value is determined by subtracting the optical density of the trichloroacetic acid filtrate from the  $C_1$  value. This is permissible since a solution of azoalbumin which is completely digested (no trichloroacetic acid precipitate) has the same color intensity as an undigested sample.

The velocity constant is calculated for the diluted solution and multiplied by the dilution factor to obtain the reaction constant for the undiluted juice.

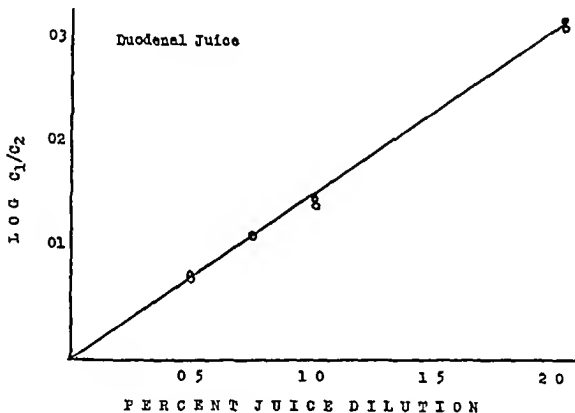


FIG 5—Relationship of proteolytic activity to the enzyme concentration of duodenal juice

*Relationship of Proteolytic Activity With Time of Digestion*—The velocity constant may be used in expressing enzyme activity only if the digestion rate of azoalbumin fulfills the requirements for a monomolecular reaction, i.e.,  $\log \frac{C_1}{C}$  should be directly proportional to time. By digesting samples of the substrate for varying periods of time with a dilution of the digestive juice the time activity relationships for duodenal juice (Fig 3) and for gastric juice, (Fig 4) were obtained. The rectilinearity of the relationship for each of the juices is in accordance with monomolecular reactions.

*Relationship of Enzyme Concentration and Activity*—The velocity constant of an enzyme reaction is a function of enzyme concentration. The relationship between enzyme activity  $\log \frac{C_1}{C}$  and enzyme concentration proved to be rectilinear in the digestion of azoalbumin with duodenal juice and with gastric juice, (Figs 5 and 6). A similar relationship was demonstrated with a

commercial trypsin concentrate.\* This direct proportionality of enzyme activity with the dilution is fortunate in that it permits recalculation to original juice or enzyme solution activity merely by multiplying the velocity constant by the dilution factor. A similar study with an aqueous extract of papaya powder fortified with cysteine did not give a rectilinear relationship except

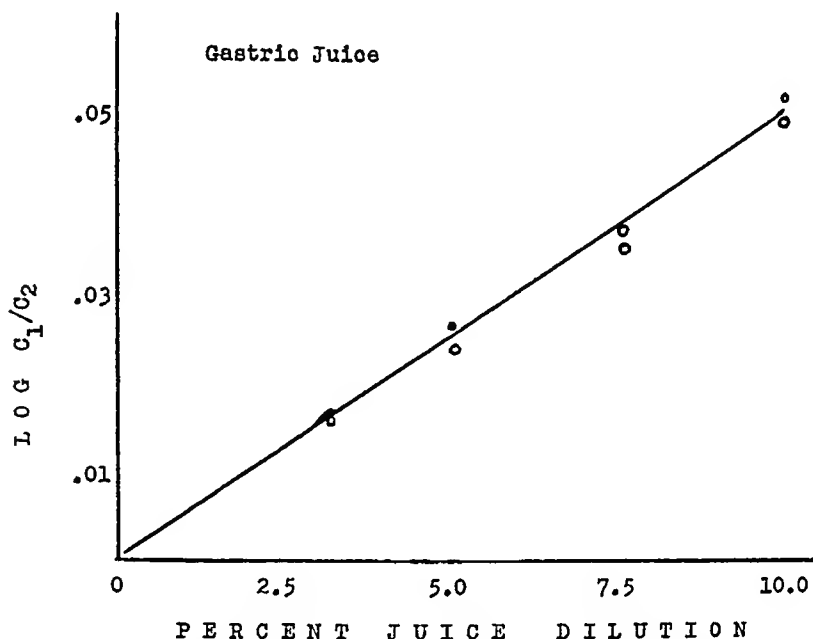


Fig. 6—Relationship of proteolytic activity to the enzyme concentration of gastric juice

in regions of extreme dilution. This nonconformity may result from the presence in the crude preparation of inhibitors which are effective at the higher concentration, or perhaps the mode of attack by papain on the azo protein differs from that of the enzymes of gastric and duodenal juice.

#### DISCUSSION

The selection of albumin for the preparation of the azoprotein substrate permits the colorimetric method for proteolytic activity to be used for gastric as well as duodenal juice. With azoalbumin as the substrate, proteolytic activity may be determined for any enzyme that is active in the pH regions above 5 and below 3. The colorimetric method has proved readily adaptable to the routine testing of clinical samples. It is simple, sensitive, and has shown a high degree of reproducibility. The substrate is easily prepared and solutions are relatively immune to bacterial contamination. Since the substrate is soluble, the method has a definite advantage in that the rate of digestion is not influenced by particle size. The color concentration relationship of azo albumin and its digestion products adheres to Beer's law, thus eliminating the necessity for nomographs. The color is stable and chromogenic reagents are not required for its development. Although the details of the cleavage of

\*Fisher Scientific Company

azoalbumin have not been studied, the method appears to be a true measure of proteolytic activity. The appearance of color in the trichloroacetic acid filtrate necessitates cleavage of the peptide bond, since the chromophoric grouping requires an intact diazotized amino acid or peptide fragment. Furthermore, comparative studies have shown that the colorimetric method has given results in good agreement with those obtained by the method of Riggs and Stadie.

#### SUMMARY

A colorimetric method for the determination of the proteolytic activity of gastric and duodenal juice is described. The digestion of a solution of azoalbumin by a dilution of the digestive juice produces colored reaction products which are soluble in trichloroacetic acid. The color intensity of the trichloroacetic acid filtrate of the digested substrate solution is a function of proteolytic enzyme concentration.

#### REFERENCES

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- 2 Riggs, B. C., and Stadie, W. C. A Photoelectric Method for the Determination of Peptic Activity in Gastric Juice, *J Biol Chem* 150: 463-470, 1943.



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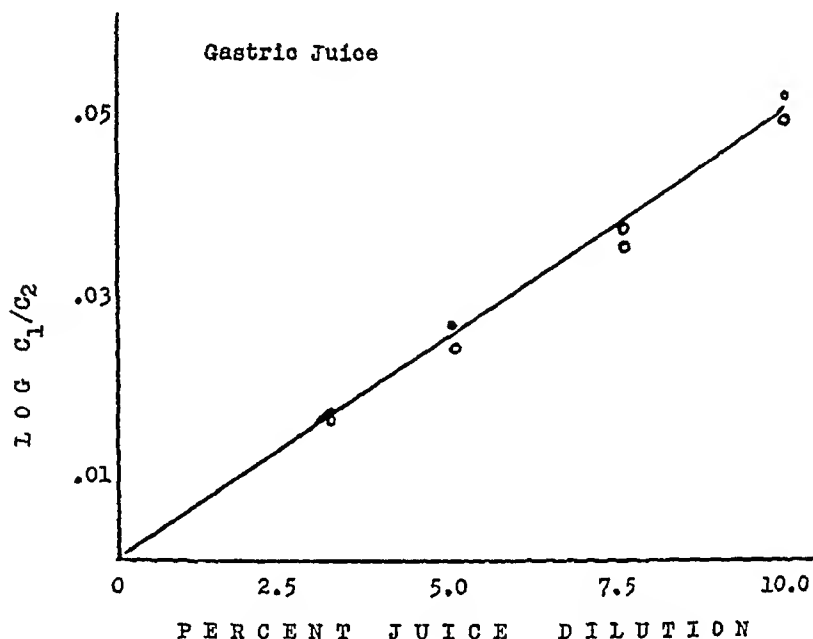


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## A HEMATOLOGIC SLIDE RULE FOR CALCULATING THE CORPUSCULAR CONSTANTS

WILLIAM R. BEST, M.D.  
CHICAGO, ILL.

SINCE Wintrobe<sup>1</sup> first demonstrated a knowledge of the corpuscular constants to be of aid in evaluating the anemias, clinicians and technicians have struggled with the necessary calculations. The time-honored and most tedious approach is through a process of long division, repeating the calculations three to arrive at mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration respectively. Those adept with the slide rule may shorten this process somewhat. Wintrobe<sup>2</sup> presents a nomogram by R. E. Mason which is easily reproduced, is reasonably accurate, and delineates normal ranges for the corpuscular constants. Its disadvantages are those of all nomograms, namely, the difficulties of deciphering which scales are used for various purposes and of fixing and maintaining a ruler on given points of widely separated scales. Whillans<sup>3</sup> has devised a circular slide rule having two scales with which the standard corpuscular constants as well as volume, color, and saturation indices may be determined. Allowance may be made for hemoglobin readings in per cent and for use of potassium oxalate alone as anticoagulant. This instrument is very useful but is not as simple nor as complete in supplementary information as is the one herein described. Recently a pharmaceutical house<sup>4</sup> has distributed a linear slide rule which will aid in various minor hematologic calculations as well as the color index, but it is not concerned with the corpuscular constants.

### DESCRIPTION

The present calculator (Fig. 1) is essentially a circular slide rule having three revolving, concentric disks graduated with appropriate segments of logarithmic scales corresponding to *C* and *CI* scales on the standard slide rule. These, together with a transparent indicator, pivot about a central rivet. The method of manipulation is simple and is clearly explained on the rule itself. Ranges of adult normal persons for red count, hemoglobin, hematocrit, and the three corpuscular constants are indicated according to the values of Wintrobe<sup>2</sup>. Approximately 85 per cent of normal persons should fall within these ranges. A scale for conversion of per cent to grams as well as scales for volume, color, and saturation indices corresponding to respective corpuscular constants is included. The latter are based on values of 5.0 million for erythrocytic count, 43.2 per cent for hematocrit, and 14.5 grams for hemoglobin.<sup>5</sup> Approximate

From the Department of Internal Medicine, University of Illinois Research and Educational Hospital.

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\*Hematorule (trade mark) 1948 The Upjohn Company, Kalamazoo, Mich.

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 ERRATUM

In the article by Chang and Hou Cold Hemagglutinin Test by a Slide Method, in the November 1948 issue of the Journal the fifth line of the Discussion, p 1449, should read ' in Table III cannot be explained by the difference in the method of examining ' ,

TABLE I RANGE OF EXPECTED ERROR OF RED COUNT USING ONE PIPETTE AND ONE COUNTING CHAMBER, ACCORDING TO VARIOUS ESTIMATES AND CALCULATIONS

TRUE ERYTHROCYTIC COUNT	EXPECTED RANGE OF ERROR		
	BERKSON	WINTROBE	PRESENT RULE
5,000,000	800,000	200,000	400,000
2,000,000	370,000	200,000	150,000

an accuracy of  $\pm 2.3$  per cent. Improperly standardized instruments may vary as much as 20 per cent. The error of the eye in matching colors in visual hemoglobinometers has been stated to be 10 per cent and is probably greater in many cases. A hematocrit after centrifugation at 3,000 r.p.m. for thirty minutes should give an accuracy of  $\pm 0.5$  per cent. In a well-regulated laboratory, Wintrobe<sup>6</sup> states that the corpuscular constants should not vary more than  $\pm 2.5$  cubic microns for M.C.V.,  $\pm 1.0$  micromicrograms for M.C.H., or  $\pm 0.8$  per cent for M.C.H.C. All of these expected ranges of variation have been roughly indicated on the calculator.

#### DISCUSSION

In checking the calculator against values obtained by long division we not infrequently have found errors of several points in the long division process.

Table II illustrates the saving in steps, and hence of time, in determining the corpuscular constants by the present method as against the other methods reviewed.

TABLE II STEPS REQUIRED IN CALCULATING CORPUSCULAR CONSTANTS ACCORDING TO VARIOUS METHODS

TECHNIQUE	NUMBER OF STEPS
Long division	30
Slide rule	9
Nomogram	9
Whillans' rule	9
Present calculator	6

Norris and Vogan<sup>2</sup> have proposed a chart showing relationship of red count, hemoglobin, and hematocrit for use in checking the accuracy of laboratory procedures against each other in routine determinations on patients without anemia and on otherwise healthy persons who have suffered acute blood loss or hemoconcentration. The calculator herein described may be used for the same purpose by setting the triangular pointers for the corpuscular constants to the 10 mark on the corresponding hematologic indices. Individual determinations of the three primary values (red count, hemoglobin, and hematocrit) for a patient fitting the foregoing description which are significantly out of alignment with the other two primary values may be considered in error.

#### SUMMARY

A new, simplified, circular slide rule for calculation of the corpuscular constants and related hematologic indices is presented. Ranges of adult normal persons as well as expected ranges of variation in determinations are clearly indicated. A method of using the instrument for checking laboratory procedures against each other is outlined.

# RESPONSE OF LINGUAL MANIFESTATIONS OF PERNICIOUS ANEMIA TO PTEROYLGLUTAMIC ACID AND VITAMIN B<sub>12</sub>

JAMES F. SCHIFF, M.D. AND R. W. RUNDLES, M.D.  
DURHAM, N. C.

ALTHOUGH the therapeutic effectiveness of synthetic pteroylglutamic acid in the majority of patients with pernicious anemia has been satisfactory, shortcomings have been observed. At the beginning of therapy, reticulocytosis although prompt is often suboptimal. The rise in red cell count is usually slower than with liver extract. Macrocytosis may persist for months, and during maintenance therapy the blood values may even decline.<sup>4, 10, 11, 13, 17, 19, 20</sup> While some patients with the neurologic manifestations of pernicious anemia may show improvement early in the course of pteroylglutamic acid therapy,<sup>5, 8, 9</sup> the failure of this compound in doses of 25 to 20 mg. per day to protect patients against neurologic relapse has been of great concern.<sup>11, 13, 14, 17, 21, 24, 27, 30, 31</sup>

The effect of pteroylglutamic acid on the third important clinical manifestation of pernicious anemia, that of atrophy and inflammation of the lingual mucosa, has received less study, but in some cases at least it appears to be unsatisfactory.<sup>11, 13, 15</sup> The purpose of this paper is to report additional patients with the lingual manifestations of pernicious anemia who were treated with pteroylglutamic acid. In some the response was good, in others poor. Two developed severe lingual relapses while taking 30 to 50 mg. per day of the compound. The lingual abnormalities in the latter cases responded to injections of vitamin B<sub>12</sub> as promptly as did the lingual manifestations in patients with pernicious anemia who had had no previous treatment.

Each of the following patients exhibited one or more of the clinical manifestations of pernicious anemia. All of them had histamine refractory gastric achlorhydria. Other complicating diseases were not present. Details of the neurologic disease in some cases will be reported later.

The following patient was one of two in whom pteroylglutamic acid therapy produced a rapid restoration of normal lingual mucosa. The tongue remained normal and there was neurologic improvement in spite of an incomplete hematologic remission.

W. P. 68799. This 55-year-old mulatto admitted to Duke Hospital on Aug. 15, 1946, first developed the lingual and anemic manifestations of pernicious anemia in 1936. He responded well to injections of liver extract but being indifferent about maintenance therapy, suffered at least three relapses, one with considerable neurologic damage. For at least six

From the Department of Medicine, Duke University School of Medicine and the Hematology Laboratory, Duke Hospital.

The pteroylglutamic acid Forte was kindly supplied by Dr. Stanton M. Hardy of Lederle Laboratories.

The vitamin B<sub>12</sub> was supplied through the courtesy of Dr. Augustus Gibson, Merck & Co., Inc.

This work was supported by a grant from the Anna A. Hanes Fund.

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## NOTE ON A SUBSTANCE TO SEAL PLETHYSMOGRAPHIC CUPS OF THE BURCH-WINSOR TYPE

C W ROBERTSON, M D, AND R H SMITHWICK, M D  
BOSTON, MASS

A MINOR problem in the use of a digit plethysmograph in which an airtight seal must be obtained between a plastic or glass cup and the digit is the choice of a sealing compound. The Burch-Winsor plethysmograph\* is an example of this type of apparatus. There are at the present time approximately eighty of these machines in use. Our experience with a new type of sealing compound therefore is considered worth reporting in the hope that it may help others in the use of this type of instrument.

The sealing compound furnished with the machine is a mixture of printers' compound and ordinary glue. Various proportions of these two substances yield products with slightly different physical characteristics with regard to melting points, rubberiness, etc. All combinations, however, have the disadvantages of (1) being water soluble (2) requiring their use in a heated state, (3) being very tacky and difficult to manage if not applied immediately after transfer from the heating bath. Being water soluble makes the compound unstable when the digit is perspiring, with dilution of the compound at the skin surface and consequent instability of the cups or even loss of seal. The last two disadvantages are of particular importance in dealing with digits which are surviving on impaired or collateral circulation entirely. In such patients use of a melted sealing compound introduces the likelihood of producing a burn in tissues that are of borderline viability.

In attempting to find a substitute, several of the commercially available cements—rubber glues, liquid adhesives—were tried. The compound which was found to be most useful was a substance located at Sears Roebuck and Company called Kalk-Koid†. This was originally designed for a number of things including repair of windows, bathrooms, sealing boats, etc. The compound was tried and is at the present time being used as follows in our laboratory. The digit is measured by placing it into a gentian solution until it has displaced 5 c.c. of solution. A ring of the compound is then placed around the finger at the top of the stain and the cup then placed over the digit to the level of the stain. A seal is obtained by working the compound around the cup with the fingers. Runs of the same digit using the commercial printers' compound-glue jelly and the Kalk-Koid at different times have shown no significant change in the pulse amplitude so that we feel the Kalk-Koid does not cause significant venous occlusion and consequent change in digit volume. This substance has shown no tendency to change consistency at the range of temperatures which we employ in our surface temperature laboratory, i.e., 65° to 85° F, and it seems to be quite insoluble in water.

From the Department of Surgery, Boston University School of Medicine.

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\*Made by the Cambridge Instrument Company, New York N. Y.

†Manufactured by the Presstite Engineering Company, 3900 Chouteau Avenue, St. Louis.

deficiency Dietary treatment was attempted without notable success Six months before admission she became markedly forgetful At that time her tongue again became red and sore Numbness and tingling in her extremities became worse Two months before admission confusion, weakness and gross unsteadiness in walking became severe enough to confine her to bed.

Examination showed a confused poorly oriented, inattentive woman barely able to move her legs in bed and just able to sit up alone The tongue was beefy red and entirely smooth except along the margins where remnants of filiform papillae were just visible Neurologic examination showed evidence of severe peripheral neuropathy, posterior and lateral column disease The hemoglobin was 13.5 grams per 100 c.c. RBC 4,020,000 WBC 19,000 hematocrit 40.8 per cent and average cell volume 101 cubic micra

Pteroylglutamic acid was given by mouth for four weeks in a daily dose of 100 milligrams After seven days the color of the tongue was nearly normal and regenerating filiform papillae were easily visible During the second week the patient became stronger and clearer mentally but then she appeared to get worse The filiform papillae which had remained stubby became shorter and the color of the tongue became a deeper red The red cell count fell to 3,630,000 and the hematocrit 35.1 per cent There was no change in her condition after five days of vitamin B therapy 30 mg of thiamin 15 Gm. para-aminobenzoic acid 10 mg riboflavin 300 mg niacin 50 mg calcium pantothenate and 5 mg pyridoxine being given daily It was then considered advisable to begin intensive liver extract therapy There was little apparent improvement during the first month except for regrowth of lingual papillae and rise in blood values After two months of liver therapy the patient's strength and motor ability began to improve steadily The hematologic values and the appearance of the tongue became normal and remained so At the end of twelve months of liver therapy she was able to do all ordinary activities at home with little motor handicap Her mental condition improved until she was about as well as before her illness Neurologic examination continued to show the expected residuals of posterior and lateral column disease

The following two patients treated with pteroylglutamic acid developed distinct lingual relapses during the third month of therapy in spite of satisfactory hematologic and neurologic responses

**C-33368** This 30 year old married postal clerk was admitted to Duke Hospital on April 26 1948 Five years previously he had developed a 'red and slick' tongue that despite vitamin B therapy became sore from time to time She became chronically weak and nervous and worried intensely about minor incidents She was afraid of "losing her mind" Nine weeks before admission to the hospital her tongue became particularly sore A few days later she found it difficult to control the movements of her feet Her legs began to feel stiff and tight Her memory deteriorated and she became confined For several days before her hospital admission she was confined to bed by extreme weakness

Examination showed a confused and apprehensive young woman She made many mistakes in doing mental arithmetic The tongue was carlet in color had no coat and in the middorsum there were no visible papillae She was unable to get out of bed without assistance and could not walk without support from both sides The tendon reflexes in upper and lower extremities were hyperactive The cutaneous senses were severely blunted below the knees There was but little ataxia with the heel to shin test The vibratory sense was absent below the costal margin The plantar responses were flexor There was sustained ankle clonus more pronounced on the left side

The hemoglobin was 12.0 grams per 100 c.c. RBC 3,300,000 WBC 8,750 hematocrit 34 per cent and the average cell volume 101 cubic micra Examination of the bone marrow showed early evidence of megaloblastic cell development

Experimental therapy with pteroylglutamic acid was started under close observation During the first month the patient was given 100 mg of the acid daily by mouth and for the next two months 50 mg daily The red cell count rose to 4,100,000 during the first month



months before his hospital admission he had again had no antianemic therapy. For three months his legs had been too weak to permit him to work. Numbness and tingling had developed about his feet and his gait had been drunken. His tongue had not felt sore.

On admission the tongue was reddish pink in color and not coated. The filiform papillae were so short as to be scarcely visible. Neurologic examination showed evidence of peripheral nerve and posterior column disease. The red cell count was 2,620,000, hemoglobin, 10.5 grams, hematocrit, 32.6 per cent, and the average cell volume, 124 cubic microns.

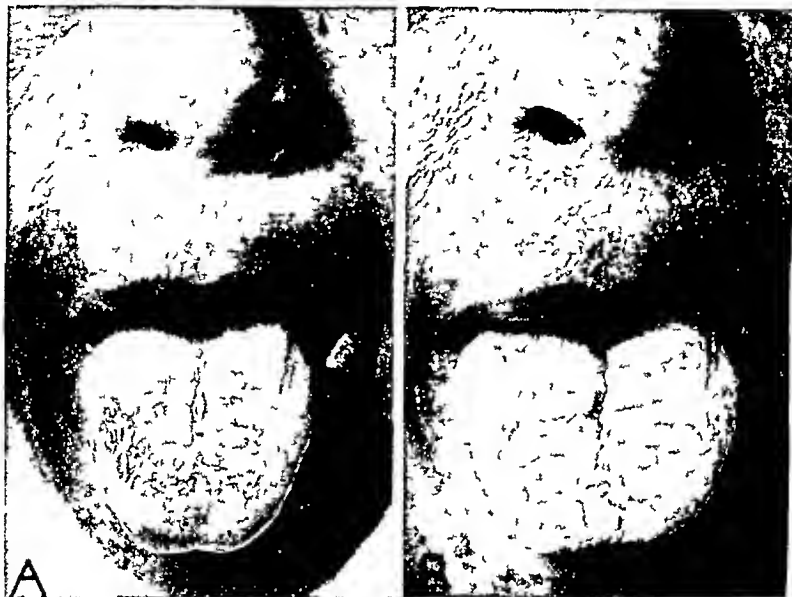


Fig 1—A, Photograph of a red smooth tongue in a patient with previously untreated pernicious anemia. B, Photograph of same tongue seven days later showing regeneration of normal lingual papillae with administration of 100 mg pteroylglutamic acid daily.

Pteroylglutamic acid was given in a dose of 75 mg daily by mouth. After six days of therapy, the tongue had become pale pink in color and faintly coated. Filiform papillae had grown to nearly normal height. The pteroylglutamic acid was maintained during the next five months, at first 30 mg per day and then 15 milligrams. The neurologic symptoms of recent onset subsided and the patient returned to work. The appearance of his tongue remained normal. The red blood count did not rise to over 3,560,000, however, and macrocytosis persisted. Because of the persistent macrocytosis, liver extract was recommended as permanent maintenance therapy.

The following patient was one of four with pernicious anemia in whom pteroylglutamic acid failed to produce an adequate lingual response. Liver extract was more effective finally in correcting the lingual and other manifestations of the disease.

E W, B 87399. This 68 year old white woman, admitted to Duke Hospital on Aug 28, 1946, had enjoyed good general health with the exception of a mild convulsive disorder of six years' duration. Three or four years before her hospital admission her tongue became intermittently red and sore. Numbness and tingling developed in her hands and feet. These symptoms led to the diagnosis of pellagra, although she was a hearty eater, her selection of food was reasonably good, and no other member of her family had symptoms of nutritional

deficiency Dietary treatment was attempted without notable success Six months before admission she became markedly forgetful At that time her tongue again became red and sore Numbness and tingling in her extremities became worse Two months before admission, confusion, weakness and gross unsteadiness in walking became severe enough to confine her to bed

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of therapy, the hematocrit to 42 per cent. After nine weeks the blood values and cell indices were entirely normal and remained so. During the first three months of treatment, her strength and ability to walk improved until she could do all of her housework without difficulty. Neurologic examination showed improved motor ability, less cutaneous sensory defect, and less pronounced ankle clonus. She gained eleven pounds in weight.

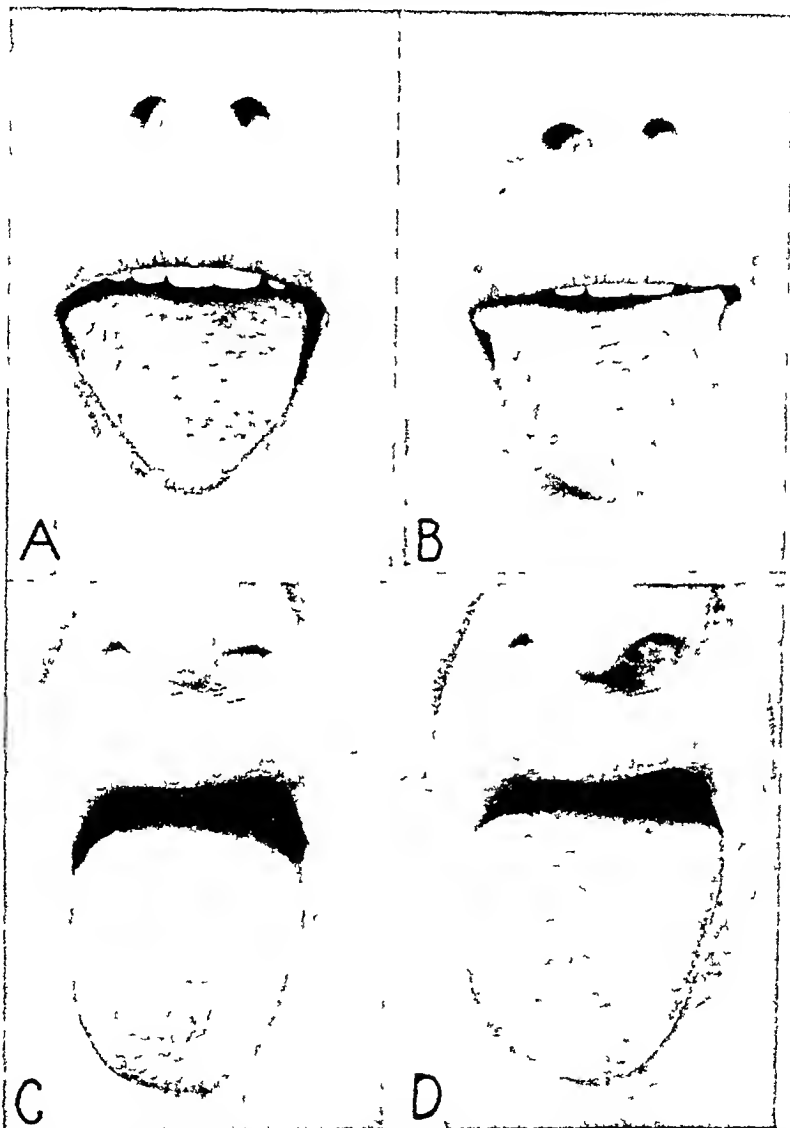


Fig. 2—A V. D. C-33368. Beefy red smooth tongue occurring as a relapse during the third month of pteroylglutamic acid therapy. B Repeat photograph showing normal lingual mucosa twenty days after the injection of 0.050 mg. of vitamin B<sub>12</sub>. C, M. J. 25597. Scarlet red smooth tongue occurring as a relapse during the third month of pteroylglutamic acid therapy. D, Repeat photograph twenty-one days later showing normal lingual mucosa after the injection of 0.025 mg. of vitamin B<sub>12</sub>.

The lingual response was never entirely satisfactory. During the first week of pteroylglutamic therapy, the tongue became more normal in color and in areas of previous atrophy filiform papillae reappeared. The papillae remained stubby, however, and an area at the tip of her tongue remained persistently red.

Early in the third month of pteroylglutamic acid therapy she complained of soreness and redness of the tongue lasting two or three days. Two weeks later these symptoms became persistent and she felt generally worse. Examination showed a beefy red tongue with out coat, completely devoid of papillae except in one small patch near the tip (Fig 2, A). There was no evidence of neurologic relapse and the blood values remained normal. The pteroylglutamic acid therapy was discontinued and 0.050 mg of vitamin B<sub>12</sub> was given intramuscularly. The redness and soreness of the tongue subsided within a week. On a check up examination twenty days after the injection the tongue was pink in color, lightly coated, and covered with filiform papillae of normal height (Fig 2, B). The neurologic status showed no objective changes.

M. J., 25597. This 58 year old farmer's widow was admitted to Duke Hospital on April 14, 1948. Two years previously she had developed a periodically sore tongue, lost her desire to eat, and had begun to lose weight. Her hands and feet became numb. She was confined to bed for several weeks by extreme weakness, but improved after injections were given by her family physician. Some months later she began to have progressive difficulty in walking, with gross unsteadiness. Two months before her hospital admission she became rapidly worse and was confined to bed, unable to stand or walk. Her family physician gave her oral medications and injections for pellagra without benefit.

On examination the patient was a thin, weak, apathetic woman appearing chronically ill. The tongue was pale and completely devoid of papillae. She was able to sit up in bed without assistance but was too weak to get out of bed, stand, or walk. The superficial sensations were impaired below the elbows and knees. The sense of motion and position was poor in the lower extremities. The vibratory sense was impaired over the lower ribs and absent at the pelvis and below. The plantar reflexes were extensor.

On admission to the hospital the hemoglobin was 6.8 grams per 100 c.c., RBC, 1,750,000, WBC, 2,360, hematocrit 19.5 per cent, average cell volume, 112 cubic micra, and reticulocytes, 5.0 per cent. During the first ten days in the hospital an incomplete, apparently spontaneous remission in the anemia occurred. There was no improvement, however, in the appearance of the tongue. After ten days of observation, the administration of pteroylglutamic acid was begun in a dose of 50 mg per day by mouth. A secondary reticulocytosis occurred. One week after the start of pteroylglutamic acid therapy, beginning regeneration of lingual papillae was evident. After eighteen days of therapy the papillae were of normal height. By this time the patient had gained strength and was able to sit up three to four hours a day as well as walk about the ward holding to furniture. After four weeks of anti-anemia therapy the blood values were entirely normal. After six weeks the dose of pteroylglutamic acid was reduced to 30 mg daily and she returned home.

General improvement continued. On a check up examination two months after the beginning of pteroylglutamic acid therapy, however, the tongue was observed to be reddened at the tip. The papillae remained tall. Five weeks later she complained of a sore, "scalded" tongue. Examination showed it to be scarlet red in color and entirely smooth (Fig 2, C). Her dietary habits were reinvestigated and it appeared that her food had been well selected and above average in nutritional adequacy. The blood values remained normal and neurologic examination showed continued improvement, certainly no relapse.

At this time the pteroylglutamic acid administration was stopped and 0.025 mg of vitamin B<sub>12</sub> was injected intramuscularly. The lingual soreness promptly subsided and after one week the color of the tongue was grayish pink and the dorsum was covered with a uniform crop of budding filiform papillae (Fig 2, D). She believed that her strength and motor ability had improved.

The following patient was one of five showing the lingual manifestations of untreated pernicious anemia in whom the injection of vitamin B<sub>12</sub> in a dose of 0.001 mg daily to 0.010 mg in a single dose led to regeneration of lingual papillae in 5-7 days.

W S, C 6035 This 66 year old white man was admitted to Duke Hospital on Aug 24, 1948 For over three years he had had periodic redness and soreness of the tongue attributed by physicians to his habit of chewing "home cured" tobacco Five weeks before admission, his hands and arms became numb, weak, and clumsy so that he had to quit work and could scarcely dress himself or write

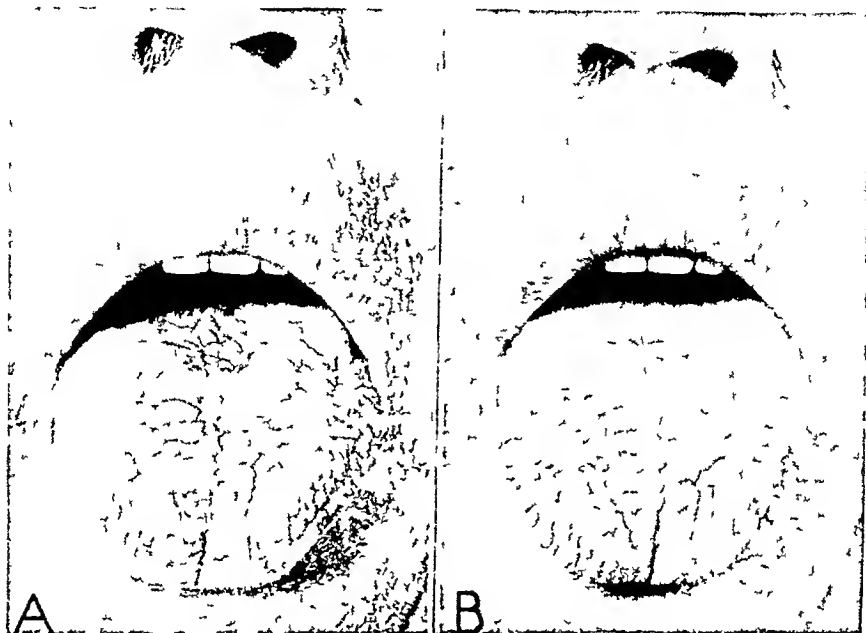


Fig 3—A W S C-6035 Dusky red tongue lacking papillae in a patient with untreated pernicious anemia B Repeat photograph five days after the injection of 0.010 mg of vitamin B<sub>12</sub> showing regeneration of papillae

On examination the tongue was deeply furrowed, completely lacking in coat, dusky red in color, and completely devoid of papillae (Fig 3, A) His grip was extremely weak Tendon reflexes in the upper extremities were absent The superficial sensations were blunted about the hands and forearms and the vibratory sense was impaired below the wrists and at the ankles The hemoglobin was 12.8 grams per 100 cc, RBC, 3,400,000, WBC, 6,800, hematocrit, 35 per cent, and average cell volume, 103 cubic micra Examination of the bone marrow showed early megaloblastic development

He was given 0.010 mg of vitamin B<sub>12</sub> intramuscularly Five days later the tongue was normal in color and its dorsum was covered with medium tall filiform papillae (Fig 3, B) in spite of his continuing to chew tobacco

#### DISCUSSION

Periodic atrophy and inflammation of the lingual mucosa, so vividly described nearly fifty years ago by Hunter<sup>15</sup> as often the first symptom of pernicious anemia, has become a well-recognized clinical manifestation of the disease Significant abnormalities of the lingual mucosa are seen, with or without hematologic or neurologic manifestations, in about one-third of present-day patients with pernicious anemia in relapse Mucosal changes of similar appearance may result, of course, from the lack of common vitamin B components in

the diet,<sup>16</sup> from iron deficiency<sup>6</sup> in association with serious hepatic disease or abdominal neoplasms, from use of oral penicillin,<sup>19</sup> etc

Among the benefits of a diet rich in liver in the treatment of pernicious anemia, Minot and Murphy<sup>15</sup> observed a gradual return of normal lingual mucosa in a matter of a few weeks or months. With potent liver extract or hog stomach therapy a more rapid lingual response occurs. Filiform papillae sprout from smooth tongues in five to seven days and attain a normal height in two to three weeks. Relapses do not occur with adequate maintenance therapy. The lingual abnormalities of pernicious anemia are not affected by nonspecific therapy, the administration of the common vitamin B components<sup>22</sup> etc. The lingual response may serve accordingly as a useful and reliable therapeutic test for pernicious anemia in patients who have little or no anemia or in whom the cause of lingual or neurologic disease is in doubt.

In the therapeutic evaluation of pteroylglutamic acid in pernicious anemia most attention has been given to the hematologic and neurologic aspects. A few investigators have noted poor lingual responses at the beginning of therapy and relapses later during maintenance.<sup>11, 13, 23</sup> The frequency of this occurrence is uncertain. It appears to be especially common in the patients reported who developed neurologic relapses. In our six patients whose lingual responses were poor or who later relapsed the blood levels remained below normal and neurologic disease progressed in one and the red cell count fell significantly in another. Had the pteroylglutamic acid therapy been continued longer in these patients a higher incidence of hematologic and neurologic failures might well have been observed.

The regeneration of lingual mucosa following the injection of vitamin B<sub>12</sub> after relapse has occurred during pteroylglutamic acid therapy shows that the former substance has a nutritional value lacking in the latter. The promptness of the response equal to that observed in patients with untreated pernicious anemia suggests that the pteroylglutamic acid failed to correct an existing deficiency rather than having exerted a positively harmful effect.<sup>7, 14, 1</sup> The effect of vitamin B<sub>12</sub> in correcting this type of lingual relapse appears to be equal to that expected from liver extract as preliminary reports indicate it to be in other respects.<sup>1, 23, 23, 6, 2</sup>

Whether or not the different manifestations of pernicious anemia—arrest in hemopoiesis, atrophy and inflammation of the lingual mucosa, and disease of the peripheral and central nervous system—represent one or more closely related nutritional deficiencies has been a perennial question.<sup>1, 2, 21</sup> Each of these manifestations is dissociated from the others and may occur singly or in combination as the disease first appears or relapses. Potent liver extracts however refined or fractionated and hog stomach preparations affect all aspects of pernicious anemia in parallel. The impression has arisen that pteroylglutamic acid corrects one portion of the basic deficiency, having an entirely satisfactory hematologic effect but being entirely ineffective regarding the neurologic manifestations.<sup>14, 1, 24, 9</sup> The incomplete hematologic remissions,

the lingual relapses, and the occasional favorable neurologic results of pteroylglutamic therapy indicate that on the contrary the compound has an irregular effect on all manifestations of the disease rather than failing consistently in any one. The cause for individual variability in therapeutic response remains to be determined.

#### CONCLUSIONS

- 1 The lingual manifestations of pernicious anemia in relapse may respond well or poorly to synthetic pteroylglutamic acid.
- 2 Two patients taking 30 and 50 mg of pteroylglutamic acid daily developed in the third month of therapy acutely sore tongues with mucosal atrophy. The lingual abnormalities disappeared within five to seven days after the injection of vitamin B<sub>12</sub>.
- 3 The lingual mucosal atrophy of five patients with untreated pernicious anemia responded in five to seven days to injections of vitamin B<sub>12</sub>.
- 4 The therapeutic limitations of pteroylglutamic acid in pernicious anemia relate to all manifestations of the disease—anemic, neurologic, and lingual—rather than to merely the neurologic.

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# CAPILLARY FRAGILITY STUDIES (GOTHLIN TEST) ON ONE HUNDRED PATIENTS RECEIVING DICUMAROL

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WHILE following the clinical course of a number of patients receiving Dicumarol therapy, hemorrhage occasionally has been found to occur without premonitory signs when the prothrombin concentration did not reach excessively low levels. Likewise it was frequently observed that the prothrombin concentration fell far below the so-called hemorrhagic levels without the development of hemorrhage. It was decided, therefore, to study a large group of patients receiving Dicumarol for varying intervals of time in order to ascertain if any correlation existed between the occurrence of hemorrhage and increased capillary fragility.

## METHOD

The modified Gothlin test<sup>1, 2</sup> was selected because of the ease of its performance, and because it has been used increasingly in many studies during the last few years in this country. Because the test has been subject to various modifications since Gothlin's original work, the method used in the present studies will be briefly outlined.

(1) Two areas, 6 cm. in diameter, were marked off in each antecubital area, noting blemishes and marks which might later be confused with petechiae. (2) A standard blood pressure cuff was applied on each arm for fifteen minutes and maintained at 35 mm. of pressure. The cuff was then released and all petechiae in each area were noted, using a good light and a magnifying lens of 5 diopters. (3) The second stage was then repeated one hour after the first, using the same procedure as previously described but maintaining the pressure at 50 mm. for fifteen minutes. (4) The petechial index was calculated as follows: To the number of petechiae in both arms occurring at 35 mm. multiplied by 2 were added the additional number of new petechiae occurring at 50 millimeters. The final index represented the sum of these two figures. Eight or less was considered normal, 9 to 12 borderline, and 12 or over increased.

Since repetition of the test before three weeks may produce false positive readings,<sup>1</sup> most patients were tested about the third week after the onset of their illness when they had received a fairly large quantity of Dicumarol.

TABLE I SUMMARY OF DATA ON NINETEEN PATIENTS RECEIVING

SEX		AVERAGE AGE	DIAGNOSES				AVERAGE NUMBER OF DAYS ON DICUMAROL	AVERAGE TOTAL DICUMAROL DOSAGE
M	F		CORONARY OCCLUSION	THROMBO PHLEBITIS	RHEUMATIC HEART DISEASE	SURGICAL PROCEDURE		
72	22	56	75	15	3	1	20	1,592 mg.

\*See Table III

†See Table IV

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Quick's method of determining the prothrombin time was used throughout the study. In this hospital the prothrombin level is always reported not only as prothrombin time in seconds but also as per cent of normal concentration. In our laboratory 12 to 14 seconds is the usual range of 100 per cent undiluted plasma, 16 to 19 seconds represents 50 per cent plasma, 22 to 26 seconds, 25 per cent plasma, and 40 to 47 seconds 10 per cent plasma concentrations.

### RESULTS

The data on ninety four consecutive patients receiving Dicumarol are summarized in Table I. Of these seventy five were being treated for acute myocardial infarction, fifteen for thrombophlebitis, three for rheumatic heart disease with embolic complications, and one for a surgical procedure.

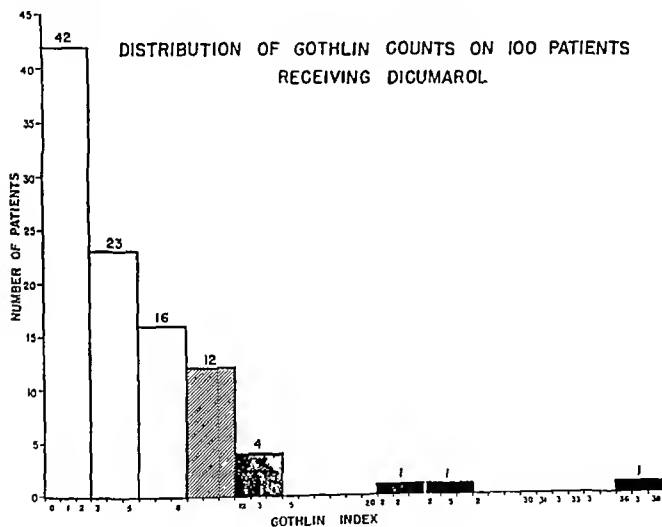


Fig. 1

DICUMAROL FOR SHORT INTERVALS STUDIED BY THE GÖTHLIN TEST

AVERAGE PROTHROMBIN CONC. DAY OF TEST	BLOOD PRESSURE		NUMBER OF PATIENTS WITH DIABETES	NUMBER OF PATIENTS WITH HEMORRHAGIC MANIFESTATIONS	NUMBER OF PATIENTS WITH POSITIVE GÖTHLIN INDICES†
	NORMO TENSIVE	HYPERTENSIVE			
27%	65	29	5	7	7

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## METHOD

The modified Gothlin test<sup>1, 2</sup> was selected because of the ease of its performance, and because it has been used increasingly in many studies during the last few years in this country. Because the test has been subject to various modifications since Gothlin's original work, the method used in the present studies will be briefly outlined.

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Since repetition of the test before three weeks may produce false positive readings,<sup>1</sup> most patients were tested about the third week after the onset of their illness when they had received a fairly large quantity of Dicumarol.

TABLE I SUMMARY OF DATA ON NINETY-FOUR PATIENTS RECEIVING

SEX		AVERAGE AGE	DIAGNOSES				AVERAGE NUMBER OF DAYS ON DICUMAROL	AVERAGE TOTAL DICUMAROL DOSAGE
M	F		CORONARY OCCLUSION	THROMBO PHLEBITIS	RHEU MATIC HEART DISEASE	SURGICAL PROCEDURE		
72	22	56	75	15	3	1	20	1,592 mg

\*See Table III

†See Table IV

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TABLE II. TREATMENT RECEIVING DURATION OF CONTINUOUS FOR LONGER DURATION

PATIENT	AGE	SEX	CONDITION FOR WHICH CONTINUOUS TREATMENT RECEIVED	LENGTH OF TREATMENT RECEIVED (mo.)	TOTAL DOSE RECEIVED (mg.)	DIFFERENCE IN GLOBULIN	PERCENT NORMAL CONCENTRATION OF GLOBULIN	COMMENT
1	20	M	Multiple pulmonary infarcts following high ligation of both femoral arteries	10 mo	50,000	0	27	
2	13	F	Multiple sclerosis	11 mo	21,000	8	22	
3	68	M	Recurrent myocardial infarction	8 mo	18,000	2	10	Two months after starting therapy with a total dose of 2,800 mg. of Dicumarol following a minor trauma, patient had ecchymosis of the skin over the ankle. Globulin test 2.1 redoubled to 10.6 normal concentration. Dicumarol continued for 6 mo. No further bleeding.
4	42	F	Recurrent myocardial infarction	9 mo	1,400	1	13	
5	65	M	Three episodes of myocardial infarction in 2 yr.	10½ mo	1,000	2	10	
6	68	M	Three episodes of myocardial infarction in 1 yr.	10½ mo	6,000	11	1	Hypertrophic stenotic blood circulation 400 mg. on first admission.

TABLE III PATIENTS RECEIVING DICUMAROL SHOWING HEMORRHAGIC MANIFESTATIONS

PATIENT	SEX	AGE	DIAGNOSIS	DAYS ON DICUMAROL	TOTAL DOSAGE OF DICUMAROL ON DATE OF DETERMINATION (MG.)	PER CENT NORMAL PROTHROMBIN CONCENTRATION ON DAY OF TEST	BLOOD PRESSURE	PAST HISTORY	GOTH LIN INDEX	HEMORRHAGIC MANIFESTATIONS
3	M	38	Recurrent myocardial infarction	240	18,300 (8 mo.)	10% 1st determination 16% 2nd determination	120/80	Two episodes of myocardial infarction in 8 mo.	2	Two months after starting therapy with a total dosage of 2,800 mg Dicumarol following minor trauma, patient had ecchymoses of skin over ankle. Gothlin test 2, prothrombin time 10% normal concentration. Dicumarol continued for 6 more mo. No more hemorrhage.
12	F	78	Coronary occlusion	15	1,650	21%	180/110	Hypertension many years	0	Gross hematuria and gross blood in stools. On 2nd day of bleeding prothrombin concentration was 43% and Gothlin remained 0. Cystoscopy showed no bladder lesion or bleeding point.
13	M	72	Coronary occlusion	15	950	12%	170/85	Recent angina	0	Gross hematuria
43	F	38	Rheumatic heart disease with auricular fibrillation, embolization, emboli	60	3,400	20%	120/80	Not significant	8	Hematuria and bilateral ureteral obstruction due to blood clots
46	M	54	Coronary occlusion	6	600	11%	100/70	Not significant	8	Gross hematuria
50	M	62	Coronary occlusion	50	2,550	22%	180/110	Angina—9 yr.	7	Two episodes of hematuria, the first on 35th day of treatment with 1,650 mg of Dicumarol, prothrombin concentration of 10% and Gothlin index of 8.
93	M	72	Coronary occlusion	24	1,650	12%	160/100	Not significant	0	Epistaxis

TABLE IV PATIENTS RECEIVING DICUMAROL SHOWING A LOSS OF GLOTHIN INDEX

PATIENT	SEX	AGE	DIAGNOSIS	DAYS ON DICUMAROL	TOTAL DOSE OF DICUMAROL (MG.)	PROTHROMBIN CONCENTRATION DAY OF TEST	BLOOD TESTS	LAST HISTORICAL ANGINA	INVESTIGATIONS	INVESTIGATIONS	INVESTIGATIONS	REMARKS
10	M	50	Coronary occlusion	23	1800	14%	100/80	Not significant	None	11	11	Dicumarol discontinued after 1 day after Gauthlin test
10	M	60	Coronary occlusion	17	1100	11%	100/80	Not significant	None	11	11	Dicumarol discontinued for 1 day after Gauthlin test
10	F	68	Coronary occlusion	11	1070	11%	100/100	Not significant	None	11	11	Dicumarol discontinued for 10 days after Gauthlin test
10	F	17	Rheumatic heart disease with murmur and fibrillation	15	1500	31%	100/60	Not significant	None	11	11	Dicumarol discontinued 1 day after Gauthlin test
10	F	70	Coronary occlusion	28	1870	16%	100/80	Diabetes	None	11	11	Dicumarol discontinued for 10 days after Gauthlin test
68	F	78	Coronary occlusion	21	1500	39%	110/80	Diabetes	None	11	11	Dicumarol discontinued day after Gauthlin test

of hemorrhage with Dicumarol, and finally some of the pathologic findings which have been observed with large doses of Dicumarol

Little data exist comparing the various methods of detection of increased capillary fragility Bell, Munio, Lazarius, and Scarborough<sup>8</sup> compared the positive pressure Gothlin test method with a negative pressure method in a group of 142 normal students They found that each test was consistent in itself, but that no correlation existed when positive pressure methods were compared with negative pressure methods They could not conclude which test was the most satisfactory for detecting increased capillary fragility In a later paper,<sup>9</sup> while studying the effects of vitamin P and C, they gave a very complete and careful analysis of all the methods of studying increased capillary fragility They observed that even in patients with frank scurvy, the capillary fragility test was negative in about one-third of their cases with the Gothlin test, and an even smaller percentage of positive reactions was found with the negative suction method Gicene<sup>10</sup> reported that 92 per cent of well-nourished children receiving supplementary vitamin C in their diets still showed a positive Gothlin test, whereas only 12.5 per cent of malnourished children with a low vitamin C intake gave a positive test He concluded that the test was not specific for the diagnosis of scurvy Beaser and co-workers<sup>11</sup> noted that with negative pressure methods contiguous areas in the same arm showed marked fluctuation in the number of petechiae from site to site Moreover, many patients had fewer petechiae at higher negative pressures than at lower pressures They therefore selected the Wright-Lilienfeld<sup>4</sup> positive pressure method for studying these cases Both diabetic and hypertensive patients showed an increased tendency to capillary fragility, the highest incidence (100 per cent) occurring when both diseases were present A considerable portion of the control group gave a positive index Increasing age seemed to play some role in the results Griffith and Lindauer<sup>12</sup> noted an increased incidence of apoplexy and retinal hemorrhage in 265 hypertensive patients studied with the Gothlin test In a larger series of 1,200 hypertensive subjects subsequently reported,<sup>13</sup> it was found that 20 per cent showed increased capillary fragility This tendency seemed to be modified by the use of rutin

Schweppe and co-workers<sup>14</sup> have compared three methods of measuring increased capillary fragility The suction or negative pressure test, the Gothlin test, and a cuff test with the manometer raised rapidly to 300 mm. of mercury Of forty-four hypertensive patients examined, 16 per cent showed a tendency to increased capillary fragility when measured by the Gothlin test and the negative pressure test, while at the same time 25 per cent showed a positive cuff test Thirteen per cent of thiocyanate-treated patients showed marked increased fragility However, as the cardiac status of the patients improved, the test often became negative even though the drug was continued Twenty per cent of the control group showed some abnormality They emphasized that no general conclusions regarding the entire capillary bed could be deducted from the reaction of the cutaneous capillaries Patients with advanced nephritis, vascular bleeding, retinitis, and ecchymoses were often encountered without the develop-

ment of a positive Gothlin test. In contrast to the findings of Sehwepe<sup>14</sup> Shanno<sup>1</sup> and Griffith<sup>12</sup> have emphasized that thioevanate therapy may predispose to the development of petechiae.

Wolfe and Danish<sup>16</sup> reported two cases of subconjunctival hemorrhage in which the Gothlin test was normal. Taylor and Page<sup>17</sup> while studying the clinical effects of rutin, using the Rumpel-Leede test noted marked variations from month to month in their control group of hypertensive patients. The test occasionally was positive in patients without cardiovascular disease. Older, acutely ill patients seemed much more susceptible to increased capillary fragility. Whereas a significant degree of arteriosclerosis gave a high incidence of positive tests, as the general status of the patient improved, particularly with improvement of congestive failure the test often became negative. The phase of the menstrual cycle also may alter the test.<sup>18</sup>

Rodrugez and Root<sup>19</sup> noted a marked increase in positive Gothlin indices in patients with diabetic retinitis. This abnormality seemed more related to the duration of the diabetes than to the chronological age of the patient. The longer the diabetes had persisted the more likely was the capillary fragility increased. Wageuer has made similar observations.<sup>20</sup> Finally, Peck and Copley<sup>21</sup> have discussed in detail physiologic factors which may influence capillary fragility and have emphasized that most cutaneous tests may not satisfactorily explain the true status of the capillary bed in more remote portions of the body.

The pathologic changes produced by Dicumarol toxicity are still not completely understood. Bingham, Meyer and Pohle<sup>2</sup> noted marked capillary dilatation in dogs receiving fatal doses of the drug. This capillary dilatation was widespread, involving not only capillaries but venules and arterioles. Frequently gross hemorrhage was noted in the gastrointestinal tract and pleural spaces. Interestingly enough the vascular walls appeared intact, hemorrhage occurring by diapedesis of red cells.

Similar findings of marked engorgement of the capillaries, venules and arterioles were found by Shevlin and Lederer<sup>3</sup> in a patient dying from uncontrolled hemorrhage after the administration of Dicumarol. Whereas hemorrhage was extremely widespread, no disassociation of the continuity of the vessel walls could be observed. They felt that the probable extravasation of blood was due to diapedesis of the red blood cells, rather than to rupture of the walls of the vessels.

In considering the patients who were on Dicumarol for long periods of time, it is particularly interesting to note Link's observation<sup>4</sup> that rabbits fed spoiled sweet clover repeatedly for seven years, resulting in a marked reduction of their prothrombin concentration for at least half of their life span, died of old age without apparent effect of the drug.

Although Barker and associates<sup>5</sup> have emphasized that hemorrhage is less likely to develop in patients receiving Dicumarol, if the prothrombin concentration is not lower than 10 per cent of normal, the literature contains many well documented cases of hemorrhage occurring when the prothrombin concentration was above the so called crucial level.<sup>3, 4</sup> Bruzelius<sup>22</sup> observed that in 28 per cent of patients with gross hemorrhage, there was no relationship between



the occurrence of hemorrhage and excessively low prothrombin concentration. It has likewise been observed by many that the prothrombin concentration of the blood often falls to levels of less than 5 per cent of normal without the development of hemorrhage.<sup>3, 24</sup> Overman and co-workers<sup>27</sup> called attention to this in some of their earlier publications stating, "The fact that hemorrhage does not always accompany drastic reductions in prothrombin concentration from a single large dose of Dicumarol, suggests that the onset of bleeding must represent more than a simple suppression of the normal prothrombin levels." Thorenson<sup>28</sup> observed hemorrhage in a patient given large doses of Dicumarol three and one-half months after the last dose of the drug. There was no correlation between the occurrence of hemorrhage and the level of prothrombin concentration. Bollman and Preston<sup>29</sup> noted that some other factor than the depression of prothrombin seemed necessary for the production of hemorrhage.

To detect and anticipate hemorrhage in patients receiving Dicumarol, careful observation of the patient and meticulous laboratory determinations must be carried out. Although, as previously emphasized, hemorrhage may occur at any level of prothrombin concentration, it is not a serious complication in careful hands. Thus, Allen<sup>30</sup> reported major hemorrhage in only 1.9 per cent of 1,686 patients receiving Dicumarol postoperatively. Barker<sup>25</sup> emphasized that definite bleeding is rare if the prothrombin concentration does not fall below 10 per cent of normal, and then it occurs in only 4 per cent of cases.

#### SUMMARY AND CONCLUSIONS

- 1 One hundred patients receiving Dicumarol were studied to determine if any correlation existed between the occurrence of hemorrhage and increased capillary fragility as measured by the Gothlin test.

- 2 Six of these patients had received Dicumarol continuously, the shortest period was three months and the longest, nineteen months. None of these patients demonstrated a positive Gothlin test.

- 3 Hemorrhage was observed seven times in 100 cases of patients receiving Dicumarol. In none of these was the Gothlin index positive.

- 4 Seven patients demonstrated a positive Gothlin test who gave no clinical evidence of hemorrhage.

- 5 The interpretation of the various tests for the detection of increased capillary fragility is briefly discussed.

- 6 The pathologic findings of hemorrhage occurring with Dicumarol are reviewed.

- 7 This study suggests that the status of the cutaneous capillaries as detected by the Gothlin test does not reflect alterations which may occur in other portions of the capillary bed.

- 8 Careful clinical observation and meticulous laboratory control are necessary to detect and prevent hemorrhage in patients receiving Dicumarol.

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# THE USE OF RUSSELL VIPER VENOM AND LECITHIN AS THROMBOPLASTIN IN THE ESTIMATION OF PROTHROMBIN

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WITH the increasing use of dicoumarin in anticoagulant therapy, methods for the estimation of prothrombin have become the subject of widespread controversy. Most published methods are based upon the one-stage procedure of Quick<sup>1</sup> in which dried rabbit brain is used as thromboplastin, but many workers have found the preparation of this reagent troublesome and have had difficulty in reproducing the reference curve whereby Quick calculates the prothrombin concentration from the clotting time. For this reason rabbit brain extract has often been replaced by Russell viper venom (Fullerton<sup>2</sup>) which is packed in a convenient form (Stypven, Burroughs Wellcome) and remains stable for long periods as a dry powder. Addition of lecithin (Hobson and Witts<sup>3</sup>) to venom gives a preparation much more active than Quick's brain extract and which appears to have all the properties of a complete thromboplastin. However, when venom reagents are used for estimating prothrombin in plasma from patients receiving dicoumarin, the results obtained are usually much higher than those found by Quick's method (e.g., Wilson<sup>4</sup>) and these discrepancies have led Macfarlane<sup>5</sup> to condemn the use of venom as likely to give a false sense of security at a time when an estimation using brain thromboplastin would reveal a disastrously low concentration of prothrombin.

It is generally admitted that the most satisfactory method for prothrombin estimation is a two-stage procedure such as that first introduced by Warner, Brinkhous, and Smith<sup>6</sup> and it is very strange that among the many one-stage methods described in the literature the results obtained have seldom been directly compared with those of a two stage method. This is probably due to the fact that the method of Warner, Brinkhous, and Smith calls for purified reagents of standard activity which are difficult to prepare and somewhat unstable. These objections were largely overcome by Herbert,<sup>7</sup> a modification of whose method has been used in the present investigation, and it has been possible by the use of this method to compare estimations of prothrombin in dicoumarin plasma by the two-stage procedure with results given by several different one-stage methods.

## METHODS

*Blood Sampling*—Decalcification was carried out by mixing 8 to 12 ml blood with 0.1 ml 10 per cent potassium oxalate, after which the blood was briefly centrifuged so that some platelets remained in suspension. The presence of platelets assists in detection of the clot in the venom lecithin method.

### (1) *Venom Lecithin Method*—

*Lecithin*—A 10 per cent solution of crude Orolecithin B P in alcohol was kept in the refrigerator.

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*Venom* A solution of 0.1 mg Styppen in 21 ml. water was stable for about a week at -12 C

*Venom Lecithin Reagent* Venom solution (0.5 ml) was mixed with  $\frac{N}{200}$  NaOH (1.5 ml) and lecithin (0.1 ml), placed in ice water and used within one hour (The concentration of the NaOH was such as to give a final pH of 7.0 to 7.4)

*Calcium Chloride* A solution containing 9.8 Gm  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  was made up to 100 milliliters. Dilution of 0.3 ml of this stock solution to 10 ml gave the 0.15 per cent  $\text{CaCl}_2$  used for the estimation. It was kept in a water bath at 38 C. This concentration is lower than that advised by Quick, who used more oxalate for decalcification of the blood.

*Apparatus* Many of the difficulties encountered in the use of venom and lecithin have been due to uncertainty in detection of the formation of the clot coupled with the fact that the clotting times are so short (normal, 6 to 8 seconds) that small timing errors are of decisive importance. A brightly illuminated water bath is essential, in which the tube must be kept immersed from the moment of addition of the  $\text{CaCl}_2$ . The bath used in this work consisted of a small accumulator jar (17 by 9 by 9 cm) standing on a black and white titration tile and filled with water at 37 to 39 C to within 0.5 cm of the top. The bath was simultaneously kept warm and illuminated by an adjustable lamp placed about 5 cm from the left side of the jar, level with the surface of the water and with the beam directed downward at an angle of 45 degrees. Glare from the lamp was prevented by a small vizard fixed to the edge of the shade.

*Procedure* Plasma (0.1 ml) and venom lecithin (0.1 ml) were mixed in a thin walled test tube (75 by 8 mm). The tube was shaken gently in the water bath for about fifteen seconds. 0.1 ml 0.15 per cent  $\text{CaCl}_2$  measured in a wide bore straight blood counting pipette was blown sharply into the tube and timing started immediately. The tube was held over the junction between the black and white portions of the tile at an angle of 10 to 20 degrees to the horizontal. The base of the tube rested on the surface of the water and the upper end was in contact with the wall of the jar just below the point at which it was held. The tube was rolled to and fro between the finger and thumb about its longitudinal axis, care being taken to maintain it at a constant angle so that the contents of the tube were not shaken. The tube was watched carefully from above until clotting occurred. The end point was the sudden appearance of a scalloped edge which was followed by a solid clot. The scalloped edge was a very precise phenomenon which could be timed with great accuracy, and was best seen in plasma containing platelets.

The method of timing is of crucial importance. Mechanical aids such as stop watches have the defect that considerable reaction time delays are involved in their use. The writer has found that the best method is to count the seconds, which eliminates errors of this nature. With a little practice it is possible to count seconds by means of a mnemonic such as "Photo graph one photograph two etc." with an accuracy of  $\pm 1$  second per minute.

*Reference Curve* The following dilutions of plasma were made with 0.85 per cent NaCl—75, 50, 25, 12.5 and 0.25 per cent. The clotting times were observed as described with the exception that the 12.5 and 6.25 per cent dilutions sometimes did not show a scalloped edge but only the sudden appearance of floccules of fibrin. The "prothrombin index" was calculated in each case as,

$$\frac{\text{Clotting time of undiluted normal plasma}}{\text{Clotting time of diluted plasma}} \times 100$$

The mean indices from ten normal specimens of plasma are shown plotted against the plasma percentage in Fig. 1. The use of the prothrombin index rather than the clotting time made it easier to read results from the curve and also avoided difficulties due to variations in the clotting time of normal plasma with different batches of venom. Although any given batch gave very consistent results, it was advisable to check the clotting time

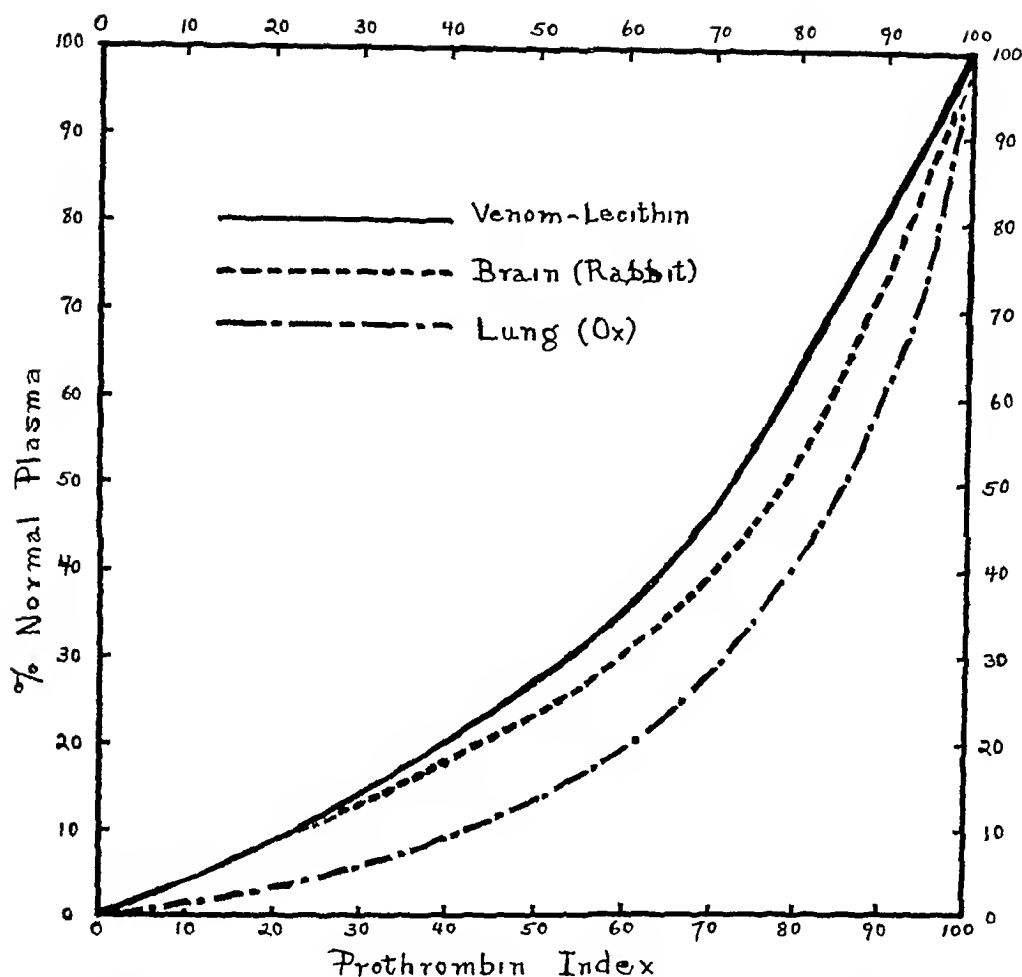


Fig 1—Variation of prothrombin index with saline dilution of normal plasma—mean of ten experiments

$$\text{Prothrombin index} = \frac{\text{Clotting time of normal plasma}}{\text{Clotting time of diluted plasma}} \times 100$$

at least one specimen of normal plasma each time a prothrombin estimation was done. The reference curves given by different batches of venom were practically identical when the prothrombin index was plotted against the dilution.

Reasons for preferring saline to prothrombin free plasma for dilution will be discussed later.

## (2) Brain Method—

**Brain Thromboplastin.** Rabbit brain was dried by the method of Quick,<sup>8</sup> finely ground, and kept in screw capped vials filled with CO<sub>2</sub> at -12° C, under which conditions it is stable for many months (Owren<sup>9</sup>). Fifty milligrams dried brain were shaken with 1.25 ml 0.85 per cent NaCl and heated at 50° C for twenty minutes. The suspension was kept at 38° and measured with a wide bore 0.1 ml pipette. Using the technique described under (1) normal plasma gives prothrombin times of 12 to 13 seconds as stated by Quick,<sup>1</sup> though the reference curve prepared by dilution with 0.85 per cent NaCl (Fig 1) differs somewhat from that calculated from Quick's published data.

*(3) Lung Method—*

An extract of ox lung was made by the method of Smith, Warner, and Brinkhous<sup>10</sup> It was heated at 50 °C for twenty minutes and kept under CO<sub>2</sub> at -12 °C. It was as stable as the dried brain, giving clotting times with normal plasma of 17 to 19 seconds when used in place of venom lecithin. The reference curve for this material shown in Fig. 1 was prepared as before by dilution of normal plasma with 0.85 per cent NaCl.

*(4) Two Stage Method—*

*Stock CaCl<sub>2</sub>* 9.8 Gm CaCl<sub>2</sub>, 6H<sub>2</sub>O made to 100 milliliters

*Saline CaCl<sub>2</sub>* 1 ml stock CaCl<sub>2</sub> made to 100 ml with 0.85 per cent NaCl

*CaCl<sub>2</sub>, Thromboplastin* 125 ml brain thromboplastin prepared as in (2) was diluted to 20 ml with saline CaCl<sub>2</sub>. The suspended particles of brain were not removed as their presence was important in detection of the clot. The mixed reagent was stable for at least a week at -12 °C.

*Barbitone Buffer* A solution of 0.6 gm sodium barbitone in 100 ml water was diluted with 350 ml 0.85 per cent NaCl and 11 ml N/1 HCl were added. The pH was adjusted to 7.3 and the solution diluted to 500 ml with 0.85 per cent NaCl.

*Fibrinogen* 30 ml unhemolyzed whole plasma were mixed with 6 ml 30 per cent suspension of BaSO<sub>4</sub>, warmed to 38 °C, and incubated for five minutes. The mixture was shaken and incubated again for five minutes. After centrifugation the supernatant was cooled in ice and 12 volumes of ice cold saturated NaCl were added slowly, with constant mixing. After standing in ice for thirty minutes the fibrinogen was centrifuged down, drained, and dissolved in 10 ml 0.1 per cent potassium oxalate. The solution was dialysed overnight at 4 °C against 300 ml 0.85 per cent NaCl containing 3 ml 5 per cent potassium oxalate and then frozen at -12 °C, under which conditions it was stable for about ten days. Before use, the solution was thawed slowly and centrifuged while a little ice remained in order to remove profibrin (Owren<sup>9</sup>).

*Procedure* Two dilutions of plasma (1:125 and 1:25) were made with 0.85 per cent NaCl, and 0.5 ml of each was placed in a test tube. To each tube was added 0.5 ml barbitone buffer and 10 ml CaCl<sub>2</sub> thromboplastin, a stop watch being started at the moment of addition of the thromboplastin. After about forty-five seconds 0.2 ml of the first (1:50) conversion mixture was transferred to a thin walled clotting tube of the type used in the one stage method. A 1 ml fibrinogen solution (0.1 ml) was blown sharply into the clotting tube, which was held in the illuminated water bath, and a note was made of the conversion time used. The counting of seconds was started at the moment of introduction of the fibrinogen and the tube was rotated and observed as in the one stage methods. The clotting time was recorded and the procedure immediately repeated with a second sample from the same conversion mixture. Successive samples were taken in this way until a clot formed in the conversion mixture which was the signal to proceed to the second conversion tube. With normal plasma the minimum clotting time was 10 to 12 seconds with the 1:50 dilution and 14 to 18 seconds with 1:100 dilution, while the corresponding conversion times were about 1 and 2 minutes. With plasma having a low prothrombin content the conversion times as well as the clotting times were considerably prolonged, but it was most unusual to encounter a conversion time for the 1:100 dilution longer than 15 minutes, with a clotting time of about 2 minutes. Hence a complete estimation could usually be completed within twenty minutes.

*Calculation* The prothrombin content of the test sample, expressed as a percentage of normal, was given by

$$\% \text{ Prothrombin} = \frac{\text{Clotting time with normal plasma}}{\text{Clotting time with test plasma}} \times 100$$

If the results of the 1:50 and 1:100 dilutions did not agree within 10 per cent, the experiment was repeated using intermediate conversion times. It was advisable to determine the clotting times with dilutions of normal plasma each time an estimation was done so that deterioration of reagents might be detected.

*Remarks* The modified two stage method just described differs in some respects from that of Herbert.<sup>7</sup> Barbitone buffer is used instead of glycylglycine and this is probably why maximum conversion occurs before instead of after clot formation. Rieben,<sup>11</sup> who also uses barbitone in a somewhat similar modification, has noticed the same difference.

Herbert's method for detection of the fibrin clot is technically difficult, but when thromboplastin containing particulate matter is used in an illuminated water bath clotting is easily detected. The fibrinogen in the conversion mixture seems gradually to accumulate on the particles of brain which move freely in the liquid as the tube is rotated. Suddenly the particles are held in a web of fibrin and the surface ceases to remain horizontal as the tube is turned, and this is the end point.

Herbert used dilutions of 1 100, 1 200, 1 400, and 1 800 in the conversion mixtures. A 1 50 dilution has been added here because plasma low in prothrombin gives inconveniently long conversion and clotting times at dilutions greater than 1 100. Results calculated from the mean of 1 50 and 1 100 dilutions were not significantly different from those obtained by the addition of 1 200, 1 400, and 1 800 dilutions.

## RESULTS

*Comparison Between Venom-Lecithin and Tissue Thromboplastins*—When comparisons were made between results of estimation of prothrombin by the venom-lecithin, brain, and lung thromboplastins it soon became evident that gross discrepancies were the rule rather than the exception. Systematic studies of patients receiving dicoumarin (Fig 2 and Table I) gave such alarming results that it was decided to follow all such cases in future by means of the two-stage procedure as well as by all three one-stage methods.

TABLE I PROTHROMBIN FOUND IN DICOUMARIN PLASMA BY ONE STAGE AND TWO STAGE METHODS

(Serial Estimations Were Made and the Figures Given Are Those Obtained on the Day When the Result by the Brain One Stage Method Was at Its Minimum)

PATIENT	METHOD			
	ONE STAGE			TWO STAGE
	THROMBOPLASTIN			
	VENOM LECITHIN	RABBIT BRAIN	OX LUNG	RABBIT BRAIN
	PER CENT PROTHROMBIN			
Le	29	4	-	-
Ow	45	16	-	-
Jo	53	12	10	-
Sp	48	30	-	52
Cr	35	14	-	48
Fu	35	24	-	57
Ta	44	25	-	35
Ev	33	9	5	11
Br	65	14	9	60
An	55	27	14	59
Wa	21	5	5	13
Ha	42	16	7	52
Me	28	21	8	27

*Comparison Between One-Stage and Two-Stage Methods*—Owen and Bollman<sup>12</sup> have recently reported that when dicoumarin is given to dogs the fall in prothrombin as measured by the two-stage method is much less rapid and profound than would appear from results obtained by Quick's one-stage technique. Six patients receiving dicoumarin have been studied throughout the course of

\*Now twenty-three patients—at time of proofreading February 1949

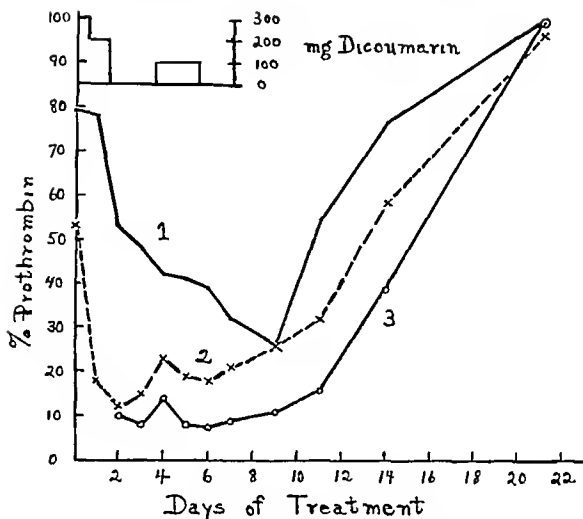


Fig 2—Prothrombin in plasma of patient treated with dicoumarin Results by one stage method using

- 1 Venom lecithin
- 2 Brain
- 3 Lung

as thromboplastin.

treatment, and it has been found in each instance that the results were similar to those observed by Owen and Bollman in the dog. One of these cases is illustrated in Fig 3 from which the results obtained with lung thromboplastin have been omitted for the sake of clarity. The one stage results obtained by the brain method were nearly always lower than those found with the two stage technique, and all the curves showed that there was reasonably good agreement between the two stage method and the one stage venom lecithin method. When ox lung was used as thromboplastin in the one stage method the results were usually much lower than those found with rabbit brain. This is illustrated in Table I in which are included results from the above mentioned six patients, together with others from patients whose prothrombin was not estimated by all four methods throughout the course of dicoumarin therapy.

*Effect of Mixing Plasmas*—When normal plasma was mixed with dicoumarin plasma the discrepancy between the prothrombin concentration as estimated by the venom lecithin and brain thromboplastins was considerably reduced. When venom lecithin was used a mixture of normal and dicoumarin plasma gave a clotting time which differed little from the "expected" clotting time calculated from the percentage prothrombin found in the dicoumarin plasma by the venom lecithin method (Table II). However, with brain



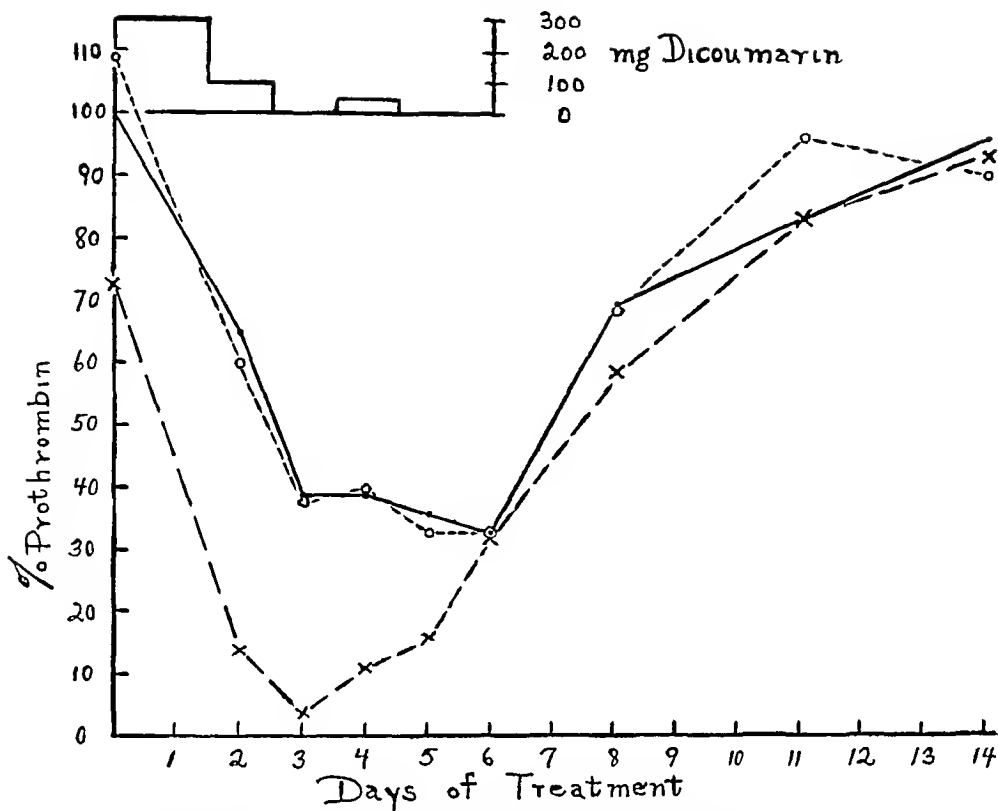


Fig 3—Prothrombin in plasma of patient treated with dicoumarin  
 One-stage method full line = venom-lecithin broken line with crosses = brain  
 Two-stage method dotted line with circles

thromboplastin, this table shows that in nearly every case the clotting time of the mixture was less than the "expected" clotting time calculated from the percentage prothrombin found in the dicoumarin plasma by the brain method. Similar results have been reported by Owen and Bollman,<sup>12</sup> using mixtures of normal and dicoumarin dog plasma, when prothrombin was estimated by the brain one-stage method. If the calculation of the expected clotting time is valid, the addition of normal plasma caused a shortening of the clotting time greater than could be accounted for by the added prothrombin as estimated by the brain method, whereas no such potentiating effect was observed when venom-lecithin was used.

*Influence of Factor 5*—Owen and Bollman<sup>12</sup> have suggested that the discrepancy between the results of the one-stage and two-stage methods is due to the slow conversion of prothrombin to thrombin in dicoumarin plasma, and believe that this is due to a deficiency of Factor 5 (Owren<sup>9</sup>) which is identical with Quick's<sup>13</sup> labile factor. Factor 5 is rapidly reduced when plasma is stored (Quick<sup>14</sup>) and its oxidation is revealed by an increase in the "prothrombin time" which occurs in spite of the fact that the prothrombin content of stored plasma remains constant for about three weeks (Waite, Guest, and Seegers<sup>15</sup>). If the discrepancy between the venom-lecithin and brain one-stage methods was due to a deficiency of Factor 5 in dicoumarin plasma, a similar discrepancy

TABLE II EFFECT OF MIXING NORMAL AND DICOUMARIN PLASMA, CLOTTING TIME (SEC) OF MIXTURES OF NORMAL AND DICOUMARIN PLASMA

VENOM LECITHIN				BRAIN			
NORMAL	DICOUMARIN	NORMAL + DICOUMARIN (1 1)		NORMAL	DICOUMARIN	NORMAL + DICOUMARIN (1 1)	
		OBSERVED	EXPECTED			OBSERVED	EXPECTED
8	11½	9	9¼	15	138	18	20
8½	13½	10	10	15	56	19	19
8	14	11½	10	15½	84	18½	19¼
8	14½	10	10	17	180	19½	22¼
9	23	12	11¾	14	57¼	15¾	16¼
8	10½	10¼	9	13¼	40¼	14¼	16
6¾	7¾	7¾	7½	13	17	13½	14

NORMAL	DICOUMARIN	NORMAL + DICOUMARIN (1 3)		NORMAL	DICOUMARIN	NORMAL + DICOUMARIN (1 3)	
		OBSERVED	EXPECTED			OBSERVED	EXPECTED
7	14	10¼	10	12½	72	17¼	21¼
7½	15½	10	11	13¼	63¼	16	23
8	12¼	10	10¼	14	30¼	17	21
8	12½	9¼	10½	14	30	16¼	10
8	9½	9¼	9	14	10	16	16¼
7½	10	9	9¼	13¼	20	15¼	16½
8	10	9¼	9	13¼	19½	15	16½
7	11	9¼	9¼	13	28	15	20
7	11	0¼	0¼	13	67	15	23
7	11½	9	9¼	12¼	36	15	18
7	13	10½	10	12¼	33	15	10

*Example of calculation of expected clotting time in an experiment using venom lecithin*

Clotting time of normal plasma 7½ seconds

Clotting time of dicoumarin plasma 15½ seconds

Prothrombin index of dicoumarin plasma =  $\frac{7.5}{15.5} \times 100 = 48$

Percentage prothrombin in dicoumarin plasma (from Fig 1) = 5 per cent.

Mixture of 1 part normal + 3 parts dicoumarin plasma should give  $\frac{100 + 75}{4} = 44$  per cent prothrombin

From Fig 1 a plasma containing 44 per cent prothrombin should have an index of 68

The clotting time of a plasma having an index of 68 would be  $\frac{7.5}{68} \times 100 = 11$  seconds

Hence the expected clotting time of the mixture is 11 seconds

should exist when stored plasma is tested by the same methods. However, the "prothrombin percentage" found by the venom lecithin one stage method with stored human plasma is nearly always lower, not higher, than the result obtained with rabbit brain. A few experiments in which dicoumarin plasma has been mixed with prothrombin free normal plasma containing Factor 5 (human plasma treated with  $\text{BaSO}_4$  and ox plasma passed through a Seitz filter) have given no evidence that dicoumarin plasma is deficient in this factor. Dicoumarin plasma will itself reduce the clotting time of stored human plasma in spite of its low prothrombin content, but results from such mixtures are difficult to interpret since each of these plasmas contains factors deficient in the other.

*Influence of Venom Lecithin on Thrombin*—The possibility existed that the short clotting times obtained with venom lecithin were due to acceleration

of the activity of thrombin. This was investigated by observing the effect of adding venom-lecithin, brain, or saline to calcium chloride and thrombin and then adding fibrinogen. The fibrinogen was prepared by adsorption of human plasma with  $\text{Ca}_3(\text{PO}_4)_2$ , precipitation with ether (Kekwick and co-workers<sup>16</sup>), reprecipitation with 12 volumes of saturated NaCl, and removal of profibrin by freezing. It contained 69 per cent of clottable protein. It was found (Table III) that venom-lecithin, brain, and also venom-lecithin plus brain all had a slightly inhibitory action on the activity of thrombin.

TABLE III INFLUENCE OF THROMBOPLASTINS ON THROMBIN ACTIVITY  
(Each Tube Contained 0.1 MI. 0.15 Per Cent CaCl<sub>2</sub> and Timing Was Started After Addition of 0.1 MI. Fibrinogen to the Mixture)

	ML. SOLUTIONS ADDED			CLOTTING TIME
	THROMBOPLASTIN	THROMBIN	SALINE	
Experiment 1	Venom lecithin 0.1	0.0	0.1	No clot 30 min
	Brain 0.1	0.0	0.1	No clot 30 min
	None	0.1	0.1	34 sec
	Venom lecithin 0.1	0.1	0.0	47 sec
	Brain 0.1	0.1	0.0	42 sec
Experiment 2	Venom lecithin 0.1	0.0	0.2	No clot 30 min
	Brain 0.1	0.0	0.2	No clot 30 min
	None	0.1	0.2	18 sec
	Venom lecithin 0.1	0.1	0.1	21½ sec
	Brain 0.1	0.1	0.1	23½ sec
	Venom lecithin 0.1 plus brain 0.1	0.1	0.0	22½ sec

Experiment 1—Thrombin 0.2 mg. per milliliter fibrinogen 0.15 per cent

Experiment 2—Thrombin 0.4 mg. per milliliter fibrinogen 0.30 per cent

The thrombin used was Parke Davis Thrombin Topical

*Prothrombin Conversion Time With Different Thromboplastins*—It has been pointed out by Warner, Binkhous, and Smith<sup>6</sup> that the prothrombin conversion time of plasma varies with the prothrombin content, and this is illustrated by the data shown in Fig. 4, all of which are taken from the records of one patient who was treated with dicoumarin for a month. These results were obtained using the modified Herbert technique with brain thromboplastin, and it seemed possible that the discrepancy found in the one-stage experiments between venom-lecithin and brain might be due to the former thromboplastin giving very much more rapid conversion of prothrombin to thrombin than the latter. Experiments were therefore done in which thromboplastins other than brain were used in the two-stage method. Table IV shows that with normal plasma, brain gave the shortest conversion times and lung the longest, with venom-lecithin occupying an intermediate position. It is also evident that at the lower dilutions the three thromboplastins produced the same amount of thrombin, so the influence of antithrombin may be discounted. The lengthened clotting times obtained with venom-lecithin at the higher dilutions were probably due to the comparative instability of this reagent. If the antithrombin effect had been of any importance it should have been

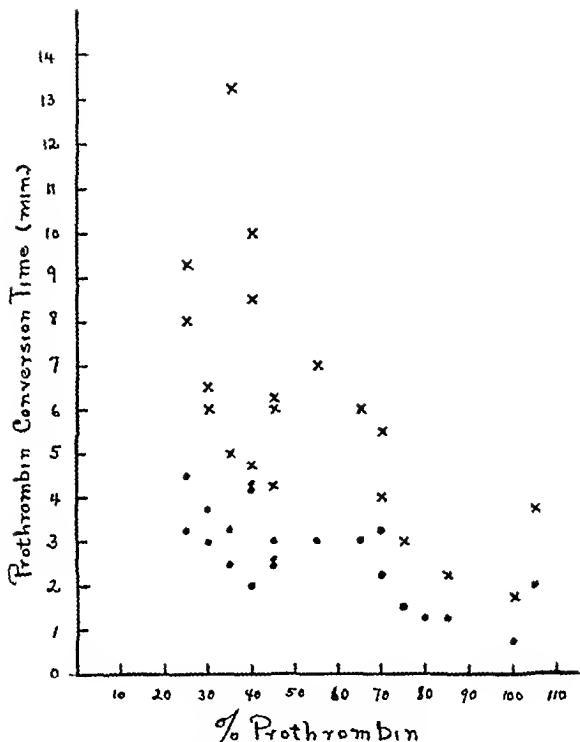


Fig 4—Variation of prothrombin conversion time with prothrombin concentration in a patient treated with dicoumarin

Conversion mixtures dots = 1:0 crosses = 1:100

seen in the 1:50 dilution, but here a  $\frac{3}{4}$  minute conversion with brain gave the same thrombin clotting time as resulted from a  $2\frac{1}{2}$  minute conversion with lung

The experiments reported in Table IV were all done with 1:16 dilutions of the three standard one stage thromboplastins and the possibility remained that different results might be obtained at higher concentrations of thromboplastins especially if normal and dicoumarin plasma were compared. This was proved not to be the case by comparing conversion times using dilutions between 1:5 and 1:20 (Table V). With both normal and dicoumarin plasma the conversion time with venom lecithin was greater than it was with brain at all concentrations of thromboplastin tested.

TABLE IV RESULTS OF TWO STAGE TEST USING NORMAL PLASMA WITH VARIOUS THROMBOPLASTINS

CONVERSION MIXTURE (FINAL PLASMA DILUTION)		1 50		1 100		1 200		1 400		1 800	
THROMBOPLASTIN		CLOTTING TIME (SEC)	CONVER SION TIME (MIN)	CLOTTING TIME (SEC)	CONVER SION TIME (MIN)	CLOTTING TIME (SEC)	CONVER SION TIME (MIN)	CLOTTING TIME (SEC)	CONVER SION TIME (MIN)	CLOTTING TIME (SEC)	CONVER SION TIME (MIN)
Plasma 1	Venom lecithin	10	1	14½	3	21	4½	37	7	83	15
	Braun	9	¾	14	1½	22	2½	41	4¾	72	9
	Lung	9	2½	14½	5	24	8½	40	10¾	73	34
Plasma 2	Venom lecithin	10½	1½	18	3¾	29	5½	68	13½	169	31½
	Braun	10½	1	17	1¾	26	3½	43½	6½	83	11½
	Lung	11	2½	18	6¾	25	11½	43	21	75	38

TABLE V RESULTS OF TWO STAGE DETIMATION OF PROTHROMBIN IN DICOUMARIN PLASMA USING VARIOUS DILUTIONS OF VENOM LECITHIN AND BRAIN AS THROMBOPLASTIN WITH A FINAL PLASMA DILUTION OF 1/160

	DILUTION OF THROMBOPLASTIN	NORMAL PLASMA		DICOUMARIN PLASMA		PER CENT PROTHROMBIN
		CONVERSION TIME (MIN)	CLOTTING TIME (SEC)	CONVERSION TIME (MIN)	CLOTTING TIME (SEC)	
Brain	1/5	23½	25	6¾	67	37
	1/75	2½	23½	9	58½	40
	1/10	2½	25	9	56	45
	1/15	3¾	26	9	52	50
	1/20	4½	26½	11	55	48
Venom lecithin	1/5	3	22	9	51	43
	1/75	3½	22	11¾	52	42
	1/10	5	24	15¾	54½	44
	1/15	6	27	15¾	73	37
	1/20	6½	26	16¾	72	36

The usual dilution of thromboplastin for the two stage method is 1/16

#### DISCUSSION

*Use of Isotonic Saline for Dilution of Plasma*—It has been shown in Fig 4 that low concentrations of prothrombin are associated with prolonged conversion times, and for this reason normal saline has been used for dilution of plasma in the preparation of standard reference curves. If prothrombin free plasma is used as advocated by many workers, conditions of rapid conversion are imposed upon mixtures of low prothrombin content which would not exist in undiluted plasma of similar prothrombin content. Table VI shows that when prothrombin is removed from plasma by adsorption with barium sulfate and the prothrombin free plasma is used as a diluent for the plasma from which it was prepared, the conversion time of the diluted plasma differs very little from that of the undiluted plasma. When plasma is diluted with saline, however, the conversion time is roughly doubled when the concentration is halved. This can be seen both in Table VI and Fig 4, and is confirmed each time a two stage estimation is performed. Inspection of Fig 4 will show that when prothrombin is reduced by dosage with dicoumarin the conversion time behaves as it does when plasma is diluted with saline, and not as it does when the dilution is made with prothrombin free plasma.

TABLE VI EFFECT OF PROTHROMBIN FREE PLASMA ON CONVERSION TIME OF NORMAL AND DICOUMARIN PLASMA (CONVERSION TIME IN SECONDS)

NATURE OF PLASMA	1/50 CONVERSION MIXTURE	1/100 CONVERSION MIXTURE
Normal	84	178
1 part normal + 3 parts prothrombin free normal	95	240
Infective hepatitis	64	101
1 part infective hepatitis + 3 parts prothrombin free infective hepatitis	95	188
Normal	125	235
Equal parts normal and prothrombin free normal	130	215
Dicoumarin	331	614
Equal parts dicoumarin and prothrombin free dicoumarin	240	500

The prothrombin free plasmas were prepared by adsorption with barium sulfate and contained no prothrombin detectable by either venom lecithin or brain one stage methods. The dicoumarin plasma had been kept at -1°C for two days.

The results obtained with dicoumarin plasma (Table VI) are interesting because this plasma had been kept at  $-12^{\circ}\text{C}$  for two days and had lost a considerable proportion of its original content of Factor 5. This is shown by the fact that the conversion times were 149 and 253 seconds for the 1:50 and 1:100 conversion mixtures when the plasma was fresh, whereas the 2-day-old plasma gave the conversion times shown in the table of 240 and 500 seconds respectively. In spite of this loss of Factor 5 (which is recognised as a substance essential for rapid conversion of prothrombin to thrombin) when the plasma was diluted to 50 per cent with prothrombin-free plasma the conversion times were rather less than those of the undiluted plasma. Since one-stage methods measure the rate of formation of thrombin and clotting may occur in normal plasma when about 5 per cent of the prothrombin has been converted to thrombin,<sup>5</sup> it is obvious that if conversion could be made instantaneous a plasma containing only 5 per cent of the normal prothrombin might give the same clotting time as a normal plasma tested in the usual way. An approach to this situation was reported by Honorato<sup>17</sup> who found that when human plasma was diluted with concentrated prothrombin-free dog plasma the "prothrombin time" remained at 11 to 13 seconds until the mixture contained less than 10 per cent of normal plasma.

Another reason for not using prothrombin-free plasma for dilution is the fact that its properties depend so much upon the method adopted for removal of prothrombin. This has been shown by Conley and Morse<sup>18</sup> and by the writer (unpublished data) who has found that if prothrombin-free plasma is prepared by adsorption with  $\text{BaSO}_4$ ,  $\text{Ca}_3(\text{PO}_4)_2$  or  $\text{Al}(\text{OH})_3$ , or by Seitz filtration, and is used for diluting normal plasma in the construction of reference curves of the type shown in Fig. 1, four different curves are obtained, each of which differs from the saline-dilution curve. Consequently, if the prothrombin index of an unknown plasma is referred to these curves five different results will be obtained for each of the thromboplastins, all of which will be lower than the two-stage result except when the index obtained with venom-lecithin is referred to a saline-dilution curve. Since there does not seem to be any satisfactory reason for preferring one form of prothrombin-free plasma to another, and since dilution with saline can give results which correspond with those of the two-stage method, dilution with saline has been used throughout this work.

*Discrepancy Between Results With Venom-Lecithin and Brain*—The reason for the failure of the various one-stage methods for prothrombin estimation to give concordant results when used with dicoumarin plasma has not been found, but certain possibilities have been eliminated. The short clotting times obtained with venom-lecithin are not due to activation of thrombin (Table III) or to acceleration of prothrombin conversion (Tables IV and V). The latter experiments are, however, only concerned with the completed conversion of prothrombin to thrombin, but it may be that with venom-lecithin the initial rate of thrombin formation is greater than it is with brain. This

is very likely, because the prothrombin time of normal plasma with venom lecithin is only half of that observed with brain and one third of that obtained with lung thromboplastin

To explain the discrepancies observed in results with dicoumarin plasma on the basis of initial conversion rates it would seem to be necessary to show that with venom lecithin the conversion rate of dicoumarin plasma is initially more nearly equal to that of normal plasma than it is when brain is used as thromboplastin. The results given in Table II are insufficient to decide this point. They show that a mixture of normal with dicoumarin plasma has a greater effect on the results with brain thromboplastin than with venom lecithin, and the only conclusion that can be drawn is that the coagulation defect of dicoumarin plasma revealed by the brain one stage method is not merely due to a deficiency of prothrombin. Assuming that the two stage method gives an accurate estimate of the prothrombin concentration, the venom lecithin one stage method described gives a closer approximation to the true prothrombin content than the rabbit brain method.

The important question remains—is the venom lecithin method suitable for control of dicoumarin therapy? The moment of clotting is more easily detected with the tissue thromboplastins and serious errors are inevitable with the venom reagent if the greatest care is not exercised. The fact that the venom reagent gives results more nearly equal to those of the two stage method than the tissue thromboplastins may be due to a happy chance but from the clinical point of view it is more desirable to estimate the tendency to bleed than to determine accurately the concentration of prothrombin in the plasma. Experience alone can show what is the danger point for any given method, and it is most unwise to apply criteria applicable to one method to results obtained with another. We believe that the important point is not the method used but the criteria employed for the interpretation of the result.

Our limited experience suggests that hemorrhage is unlikely if the plasma prothrombin as estimated by the venom lecithin or two stage methods is kept above 30 per cent of the normal value. In one instance a patient bled from a small ulcer in the mouth when the prothrombin by venom lecithin and two stage methods was about 60 per cent but in this case the result by Quick's method was 34 per cent. The latter figure is well above the accepted danger level for the brain method, which shows that caution must be observed whatever the "prothrombin time" may be. This is the only hemorrhagic incident which we have observed among eighteen patients controlled by means of the venom lecithin method and it was not sufficiently serious for the patient to notify her doctor until the bleeding had ceased. One other patient had a small hematoma of doubtful origin on her eyelid at the same time as a needle hematoma. Our clinical colleagues believe that satisfactory results can be obtained with a prothrombin concentration of 40 to 50 per cent as estimated by the two stage and venom lecithin one stage methods so we have had no opportunity of studying cases where the prothrombin had been reduced to



hemorrhagic levels. However, the tendency to bleed is sometimes not closely correlated with the prothrombin concentration and we must understand more about other clotting factors before we can control anticoagulant therapy with complete confidence.

#### SUMMARY

1 Modified methods and apparatus are described for the estimation of prothrombin by the one-stage and two-stage procedures.

2 When plasma from patients treated with dicoumarin was used, the two-stage method gave results in fair agreement with those obtained by a one stage method in which Russell viper venom and leechin was used as the thromboplastin. When rabbit brain or ox lung was used, the prothrombin concentration found was lower than that given by the two-stage method.

3 Possible reasons for the discrepancies are discussed together with the significance of these observations in the control of anticoagulant therapy.

My grateful thanks are due to Dr R G Macfarlane and Dr R Biggs for opportunities for discussion of this work.

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tion The degree of reduced prothrombin activity must be of such magnitude that it will prolong the whole blood clotting time, hence, it must be less than 40 per cent of normal activity before the titration is increased Based on experience with the normal dog given Dicumarol, the protamine titration does not increase until the prothrombin activity is less than 25 per cent of normal It may be that some revision of these data will be required in cases of reduced prothrombin activity in the presence of liver disease, as this aspect of the problem has not been investigated Normal or near normal prothrombin activity has been interpreted to indicate lack of any marked disturbance of the accelerator factor (accelerator globulin, prothrombin A, factor V)

*Heparinization* The therapeutic intravenous heparinization (35 to 50 mg per single total dose) markedly increases the clotting time but at these levels does not appreciably affect the protamine titration in otherwise normal blood If, however, the protamine titration scale is made more sensitive, the effect of heparin therapy can be detected readily Moreover, the patient with prothrombin deficiency given heparin may show an increased titration when neither the prothrombin deficiency nor the heparin alone is capable of increasing the protamine titration

*Hemophilia* Since in this disease the clotting time is prolonged, the protamine titration also may be increased This is probably a function of thrombin formation Not all hemophilic persons show an increased protamine titration, but in severe exacerbation the titration is generally increased

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*Thrombocytopenia* The titration in our experience has been more frequently normal than abnormal in idiopathic thrombocytopenia However, patients with thrombocytopenia and increased protamine titration have been clinically improved and then titration returned to normal without evidence of any increase in the platelet count<sup>2</sup>

The magnitude of the changes produced in the protamine titration by the factors listed has been minimized by (1) the range of protamine used (increase of 0.02 mg per tube), (2) brisk shaking of tubes to initiate clotting, and (3) reading the titration no sooner than one hour after mixing heparinized samples in protamine tubes

#### SUMMARY

A method for measuring a clotting defect found in certain hemorrhagic states has been described

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Two unexplained exceptions have been observed. These did not clot with 0.14 mg. protamine at the end of one hour but did clot into the normal range at the end of two hours. In a similar series of normal dogs the end point was found to be 0.12 mg. of protamine. Hereafter, the human normal end point is the one described. In women this end point may be increased during the menstrual period. Normally the clot appears firm and retracts in all tubes containing 0.14 mg. of protamine or more and is entirely fluid in all the tubes containing 0.12 mg. or less after one hour. Clot retraction, however, is slightly impaired in the tubes of high protamine concentration. The effect of other mild to moderate blood deficiencies on reading the end point is minimized by reading at one hour. This is especially true of thrombocytopenia and prothrombin deficiency.

### 3 Sources of Error

- (a) Moist or unclean glassware or needles
- (b) Inaccurate measurement of protamine and/or heparin solutions
- (c) Poor venipuncture technique. In all coagulation studies it is imperative that the blood flow is prompt and free.
- (d) Failure to age protamine solution for twenty-four hours before use using protamine solution that has been standing for longer than one week or that has not been kept refrigerated. The antiheparin activity can be checked by running a protamine titration on normal blood with each new batch of protamine solution.
- (e) Biologic variations that may occur in preparation of heparin. It is advisable to check each new vial of heparin with the previous vial by running a protamine titration on normal blood using heparin from both vials before the supply from the one in use is exhausted.
- (f) Failure to obtain adequate mixing of either heparin with the original blood or of the heparinized blood with the protamine solutions. In the latter case clotting may appear sporadically in the series of protamine tubes.
- (g) Gelation. In some cases gelation precedes or substitutes for coagulation. This problem is best resolved by reading, as the end point, the tube in which gelation first appears.
- (h) Undiagnosed hemophilic or prothrombin deficient bloods. These conditions may increase the protamine titration; the complications are described in detail later.
- (i) Clotting within normal range (0.14 mg. of protamine) after one hour. As previously stated, normal blood and most abnormal blood specimens show no further clotting after one hour. An occasional blood sample, however, may show an increase in the protamine titration at the end of one hour but clot within the normal tube (0.14 mg. of protamine) after several hours. It has been our experience that these otherwise normal individuals show no significant clotting abnormality. In a few instances a similar decrease in the protamine titration occurred in bleeding cases but these generally did not return to the normal range.

*Factors Affecting the Protamine Titration*—Interpretation of the protamine titration depends upon complete evaluation of all the known factors concerned with hemostasis. Normal hemostasis involves at least three systems: (1) vascular, (2) cellular elements, (3) plasma. Of the cellular elements the platelets are probably the most important. They are concerned with thromboplastin and are intimately associated with the plasma factors which include prothrombin, fibrinogen, prothrombin accelerator factors, antihemophilic globulin, clotting inhibitors, and possibly others not yet known.

The protamine titration is independent of the platelet count and vascular components. When the protamine titration is increased, thrombocytopenia and vascular disturbances are frequently present. This association, however, is not one of cause and effect.

The protamine titration can be markedly influenced by any factor which is capable of prolonging the whole blood clotting time.

*Prothrombin Deficiency* Prothrombin deficiency of severe degree, in both dog and man, will increase the protamine requirement in the protamine titra-

This test is not necessarily a measure of heparin itself or even of heparinoid materials, for it may be increased by several factors, only some of which are known. Hence, it is only of value when all the known factors related to hemostasis can be evaluated. It appears to be a measure of a clotting defect similar to, though not identical with, that produced by the intravenous administration of a commercial beef heparin.

### *The Protamine Titration —*

#### *1 Reagents and Equipment*

- (a) Protamine (saline) sulfate stock solution 50 mg of powdered protamine sulfate (Lilly) are dissolved in distilled water and made up to 50 ml in a volumetric flask. Do not use for twenty four hours. Keep refrigerated at 1 to 4° C (it is stable for one week)
- (b) Liquid commercial heparin (Abbott)
- (c) Ten serology tubes (1 cm inside diameter by 8 cm long)
- (d) Conical centrifuge tube graduated to 15 milliliters
- (e) 10 ml pipette, graduated to tip
- (f) 20 ml syringe, glass tipped, with thin coating of light mineral oil
- (g) 18 gauge needles, 1½ in long. These must be sharp and should be cleansed with Haemasol\* and checked with hydrogen peroxide for evidence of peroxidase before drying with alcohol and ether.

*All glassware must be chemically clean and dry before using*

#### *2 Procedure*

- (a) Pipetting the protamine solution. To each of the ten serology tubes the protamine sulfate solution is added from a micropipette in increments of 0.02 ml (0.02 mg) beginning with 0.02 ml (0.02 mg) in the first tube and ending with 0.20 ml (0.20 mg) in the tenth tube. The entire ten tube range runs serially from 0.02 ml to 0.20 ml (0.02 mg to 0.20 mg)
- (b) Pipette heparin into the conical tube. With a micropipette deliver 0.10 ml (10 mg) liquid commercial heparin to the bottom of the conical centrifuge tube.
- (c) Venipuncture. Cleanse skin with alcohol and dry. Apply tourniquet and make clean needle puncture. Gently aspirate (preferably by venous pressure) 12 to 14 ml of blood. If a good venipuncture is not accomplished on the initial puncture, another needle should be used as tissue juice or small amounts of blood may introduce serious error. The syringe should be free of air bubbles.
- (d) Mixing the blood and heparin. Remove needle from syringe and allow the blood to run gently down the side of the conical tube containing 10 mg (0.1 ml) of heparin, bringing the blood to the 11 ml mark. Stopper the tube with rubber stopper and slowly invert fifteen times to obtain reasonable mixing. The remainder of the test may be done in the laboratory and should be completed within one hour. If not pipetted at once, inversion mixing is repeated immediately before use.
- (e) Pipetting the blood. The 10 ml pipette is carefully filled with the heparinized blood mixture, avoiding aspiration of air bubbles. One milliliter of blood is allowed to flow down the side of each of the ten tubes containing the protamine solution. Each tube is shaken briskly eight to ten times to obtain reasonable mixing of the heparinized blood with protamine solution. The entire series of tubes is then allowed to stand undisturbed for one hour at room temperature before the end point is read. The end point is defined in terms of the protamine content of the tube containing the least amount of protamine in which a solid clot has formed at this time.
- (f) The end point. In a series of 100 normal male subjects all tubes containing 0.14 mg or more of protamine clotted. Those tubes containing 0.12 mg or less remained fluid.

\*Distributed by American Hospital Supply Corp. Chicago, Ill.

## ENZYME STUDIES ON HUMAN BLOOD

### III EFFECT OF PLASMA PROTEINS ON COAGULATION\*

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In a previous report<sup>1</sup> it was demonstrated that the level of the clotting times becomes elevated as the purity of the fibrinogen preparation increases. This observation prompted us to study the effect of proteins added to the substrate on the thrombin-fibrinogen reaction time.

#### METHODS AND MATERIAL

Fibrinogen fractions of 79 to 80 per cent purity were prepared from dried human plasma by the application of the low salt low temperature ethanol principle.<sup>2</sup> The hemoglobin solution was prepared by dialysis in citrate phosphate buffer (ionic strength 1/2, 0.129, pH 7.2) of lysed human erythrocytes previously washed four times with 6 vol of saline. All stock solutions, including those of Fractions II, III, IV-1, IV-4, V (Cohn<sup>3</sup>), and heated 25 per cent human albumin<sup>4</sup> (Cutter Laboratories), were made up in citrate phosphate buffer on the day of the experiment. When adjustment to pH 7.2 was necessary, 0.1M NaOH in citrate phosphate buffer was added, volume not exceeding 10 per cent of the total volume of the stock solution. Each protein solution in at least four different concentration levels was added to fibrinogen fraction solutions at final substrate concentration of 0.5, 0.25, 0.125, 0.062, and 0.031 per cent. A control series without added protein was also tested. Thus, for one experiment, duplicate or triplicate clotting times were determined on each of at least twenty-five substrates. Actually, eleven experiments involving two hundred and seventy-five test solutions with primary plasma fractions added and fifty-five control solutions with fibrinogen fraction alone were done. In addition, fifteen test solutions with varying proportions of Fraction V (albumin) and a globulin mixture of Fractions II, III, IV-1 and IV-4 were investigated. The clotting times were determined upon addition of 0.2 c.c. thrombin (a 1 to 10 dilution of Lederle's Hemostatic Globulin in citrate phosphate buffer) to 0.8 c.c. of the substrate equilibrated at 37.5° C. The reagents and techniques for clotting time and protein determinations have been described previously.<sup>1</sup>

#### RESULTS

The data on the effect of added protein on the thrombin-fibrinogen reaction at 0.25 per cent fibrinogen fraction concentration are presented in Fig. 1.

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Plasma Fractions II, III, IV-1 and IV-4 were obtained through the courtesy of Dr. E. J. Cohn. They were prepared in collaboration between the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., and the Division of Biologic Laboratories of the Massachusetts Department of Public Health from blood collected in collaboration with the American Red Cross. Fibrinogen fractions were prepared in this laboratory from dried normal plasma processed from blood obtained from volunteer donors enrolled by the American Red Cross.

tion The degree of reduced prothrombin activity must be of such magnitude that it will prolong the whole blood clotting time, hence, it must be less than 40 per cent of normal activity before the titration is increased Based on experience with the normal dog given Dicumarol, the protamine titration does not increase until the prothrombin activity is less than 25 per cent of normal It may be that some revision of these data will be required in cases of reduced prothrombin activity in the presence of liver disease, as this aspect of the problem has not been investigated Normal or near normal prothrombin activity has been interpreted to indicate lack of any marked disturbance of the accelerator factor (accelerator globulin, prothrombin A, factor V)

*Heparinization* The therapeutic intravenous heparinization (35 to 50 mg per single total dose) markedly increases the clotting time but at these levels does not appreciably affect the protamine titration in otherwise normal blood If, however, the protamine titration scale is made more sensitive, the effect of heparin therapy can be detected readily Moreover, the patient with prothrombin deficiency, given heparin may show an increased titration when neither the prothrombin deficiency nor the heparin alone is capable of increasing the protamine titration

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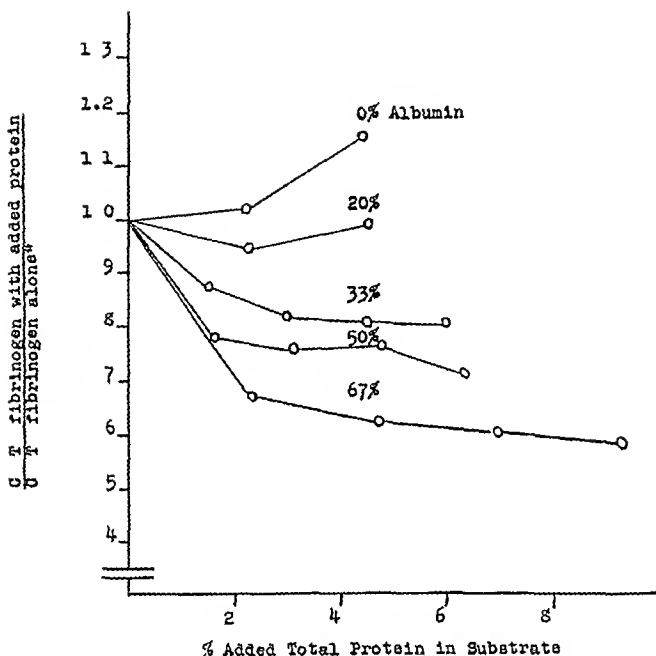


Fig. —The effect of added plasma proteins of varying albumin globulin proportions on the thrombin-fibrinogen clotting time  
 Citrate phosphate buffer 1/100 pH 7.2 0.25 per cent fibrinogen fraction in substrate  
 Clotting time 17.4 to 18 seconds

#### DISCUSSION

The foregoing experiments demonstrate without doubt that albumin has an accelerating effect on the thrombin-fibrinogen fraction clotting time. That this effect might be due to a contaminating enzyme or globulin in Fraction V is untenable for obvious reasons. Similar quantitative results were obtained with albumin prepared by the reprecipitation of Fraction V and subsequent heating of the resulting 97 to 99 per cent albumin for ten hours at 60° C in the presence of the sodium salt of acetyl tryptophan.<sup>3,4</sup> This interesting relationship of albumin to the thrombin-fibrinogen fraction reaction merits further study. There is no justification from the data on hand to state that albumin influences thrombin activity per se for it is possible that the fibrinogen molecule itself might be involved, that a coagulant contaminant might be activated, or that there may be inhibition of anticoagulant substance, if present, in the fibrinogen fraction.

It must be stated that our results do not necessarily refute the opposite findings of Quick,<sup>5</sup> who suggested that a blood albumin fraction is antithrombic and is synergistic with heparin. Extensive and careful work by other investigators has essentially confirmed this concept.<sup>6</sup> Quick<sup>5</sup> clearly emphasized that this activity might well be a contaminant in the albumin fraction. This was confirmed by several workers,<sup>7, 8, 9</sup> who demonstrated that the antithrombic activity resides in albumin fractions but not in crystalline albumin. Moreover, previous workers were purposefully testing the effect of albumin fractions on thrombin as indicated by their incubation of the two substances before reaction with fibrinogen or plasma. In our experiments the protein was added to fibrinogen fraction solutions, and no significant changes were demonstrated when clotting times were done during a three-hour period after mixing. Another reason that our results cannot be adequately compared with those of previous investigators is that the methods for the preparation of the protein fractions were not the same. Finally, the fibrinogen fractions used in this study contained approximately 20 per cent globulins (other than fibrinogen). Antihemophilic globulin<sup>10</sup> and an active proteolytic enzyme<sup>11</sup> have been reported in fibrinogen fractions.

Fraction II-III definitely increased the thrombin-fibrinogen fraction clotting time. Deutsch<sup>12</sup> determined thrombin by the two-stage method on this prothrombin-rich Fraction II-III and concluded that the assay is difficult because of the presence of thrombin-inhibitors, which he states are of two possible types: one which inhibits prothrombin conversion and another which inactivates thrombin. Since in the present study calcium and thromboplastin were not added, it would seem that only the latter type of inhibitor could apply as a possible explanation. Deutsch and Gerarde<sup>13</sup> also found that gamma globulins, as well as albumin, do not alter the clotting times of rabbit plasma with added calcium and thromboplastin, beta globulins, however, were found to have a definite retarding effect. As with Fraction II-III in this study, Fraction IV-1 caused an elevation in the thrombin-fibrinogen clotting time. Fraction IV-4 caused a slight depression in the clotting time, this may be explained on the basis of its relatively high albumin content of approximately 15 per cent.<sup>2</sup> In general, our results would indicate that the net effect of native plasma globulins in normal distribution is to increase the thrombin-fibrinogen fraction reaction time.

The experiments with albumin-globulin mixtures of varying proportions substantiate the results from experiments on individual fractions and suggest a homeostatic balance between albumin and globulins within a wide range in respect to the fibrinogen fraction-thrombin reaction.

#### SUMMARY

In a system of fibrinogen fractions (79 to 80 per cent purity) in citrate-phosphate buffer (pH 7.2, 1/2, 0.129), albumin definitely lowers, Fractions II-III and IV-1 elevate, and Fraction IV-4 and hemoglobin slightly depress the clotting times. The significance of these findings with fibrinogen fractions prepared by the low salt-low temperature ethanol principle is discussed.



The valuable technical assistance of Mary Beth Everhart is gratefully acknowledged

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## THE USE OF URINARY PIGMENT EXCRETION FOR THE MEASUREMENT OF BASAL METABOLIC RATE

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THE diagnosis and control of hyperthyroidism depends, to a large degree, upon an accurate determination of the basal metabolic rate. It is known that such conditions as neuromuscular asthenia, alcoholism, pregnancy, and apprehension may cause an increase in oxygen consumption which does not reflect the true basal metabolism. In pediatric practice, and in patients without teeth or with perforated eardrums, it is virtually impossible to obtain accurate B.M.R. determinations with the usual clinical equipment. The existence of these conditions which produce an increase in oxygen consumption rate, in the absence of an increased basal metabolism, frequently renders the accuracy of the B.M.R., as determined by the respiratory calorimeter, open to question. Since the determination of the true basal metabolism, and not a machine-measured increase in oxygen consumption, is of great importance in the diagnosis and control of thyrotoxicosis, the presence of any of the afore-mentioned conditions frequently makes it impossible to determine true basal metabolism, with our present methods. The elaboration of a clinically applicable procedure, which would serve as a measure of basal metabolism, independent of oxygen consumption, is, therefore, of great importance. Early in 1948, Dr. Irwin Slater brought to our attention the work of Ostow and Philo, on the relation of the pigment/creatinine ratio to basal metabolic rate. We investigated the accuracy and constancy of this relationship, in an effort to obtain a relatively simple yet practical and accurate measure of true basal metabolism.

The yellow color of the urine is generally ascribed to the pigment urochrome. The chemical constitution and the nature of the precursors of urochrome are unknown, although there is evidence<sup>1</sup> that tryptophane and kynurenin are involved in its production. Diabkin<sup>2</sup> observed that the output of urinary pigment is remarkably constant from day to day under ordinary conditions of health. This output is independent of diet, but bears a relation to the level of basal oxygen consumption, and is, therefore, a product of endogenous metabolism. In hyperthyroidism, fevers, and in artificially produced elevations of metabolism by the administration of thyroxin or epinephrine, the output of urinary pigment is increased. Urochrome excretion also is enhanced by tissue breakdown, starvation, or by the administration of acids. Diminished urinary pigment excretion is observed after extirpation of the thyroid, and the administration of alkalis.

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The daily creatinine excretion is extraordinarily constant for the individual and is not influenced by ordinary exercise or by urine volume. Creatinine output varies from 1.5 to 2.0 Gm daily for men and from 0.8 to 1.5 Gm for women, corresponding to about 2 per cent of body creatine content. The creatinine coefficient

$$\frac{\text{Mg creatinine excreted/day}}{\text{Body weight in kilograms}}$$

is from 20 to 26 for the majority of normal men and from 14 to 22 for women. Its value depends upon the muscular development of the individual; the sex variation is due presumably to the different relative amounts of fatty and muscular tissues of male and female bodies. Athletic women for this reason have a coefficient as high as or higher than men of obese build and poor muscular development.<sup>3</sup> Unlike the excretion of urea which is derived largely from exogenous sources, the creatinine output is practically independent of the protein level of the diet and is therefore considered to be a quantitative index of tissue and more especially of muscle metabolism.

Ostow and Philo<sup>4</sup> reinvestigated the nature of the relationship between urinary pigment output and the basal metabolic rate. To correct for weight, height, habitus, and age of the subjects, the rate of pigment excretion was compared with the rate of excretion of creatinine, the latter being constant for each individual. Since this is a ratio of the concentration of the two substances, there is no necessity for measuring urinary volumes per unit of time. This ratio (pigment/creatinine) was found to parallel the basal metabolic rates of patients to whom thyroid extract was administered. The test cannot be done in the presence of bile or free hemoglobin in the urine such as may occur during menstruation, nor following the ingestion of foods such as beets and rhubarb or large quantities of riboflavin or dyes which color the urine. In cases of renal disease with nitrogen retention<sup>2</sup> and in cases of severe liver damage and in some cases of chronic heart disease it is impossible to obtain a valid pigment/creatinine ratio. From a practical standpoint, however, these conditions do not usually coexist in patients in whom it is of importance to obtain an accurate basal metabolic rate determination for diagnosis or management.

The purpose of this investigation was to determine whether there existed an accurate correlation between basal metabolic rates as determined by the respiratory calorimeter and the pigment/creatinine ratio and if possible, to obtain a regression equation from this relationship. If a statistically valid equation could be derived, the pigment/creatinine ratio could then be applied wherever the accuracy of the basal metabolic rate measurement was in question.

#### METHOD

Patients selected for this study were instructed to report to the clinic as soon as possible after arising. On arrival they voided urine and this specimen was discarded. After resting for one hour a BMR determination was done with the respiratory calorimeter. The pigment/creatinine ratio then was determined on a urine sample obtained after the completion of the BMR test. Only those cases in which the BMR was consistent with the clinical diagnosis

were included in this study. To prevent variations in the apparent concentration of urinary pigment due to chemical alteration after voiding, all samples of urine were placed immediately in a refrigerator, and spectrophotometric readings were made as soon as possible, though never later than two hours after voiding. The urine sample is centrifuged at high speed for ten minutes to remove any sediment. A portion of the supernatant urine is then placed in a 10 by 75 ml cuvette and the per cent light transmission at a wave length of  $420\text{ m}\mu$  is determined with a Coleman Junior spectrophotometer.

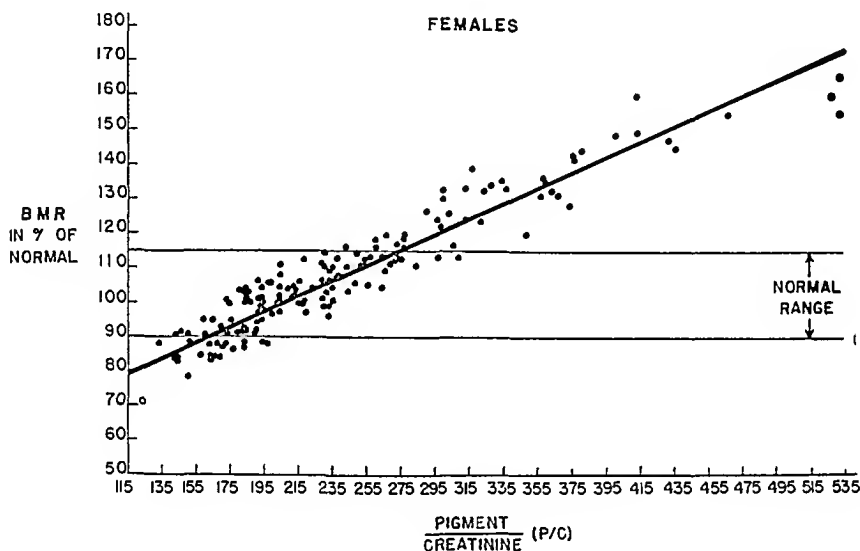


Fig 1

The urinary creatinine content is determined by a modification of the Jaffe reaction<sup>6</sup>. Urine, 0.1 ml, diluted 1:5 with distilled water (1:4 in urines of low specific gravity), 2 ml of saturated picric acid, and 0.15 ml of 10 per cent sodium hydroxide are placed in the 10 by 75 ml cuvette. After standing for ten minutes, the per cent light transmission at a wave length of  $520\text{ m}\mu$  is determined in the spectrophotometer. The creatinine concentration is determined from a graph made by plotting per cent light transmission against various dilutions of a standard creatinine solution.

## RESULTS

Determinations obtained from one hundred fifty-six adult female subjects are indicated in Fig 1, and from fifty-seven adult male subjects in Fig 2.

It will be noted in Figs 1 and 2, that the great majority of both female (ninety-four cases) and male subjects (thirty-three cases) exhibit basal metabolic rates generally considered to fall in the normal range (15 per cent above and 10 per cent below the calculated normal). From a statistical standpoint the number of cases above the normal range (forty-one female subjects, seventeen male subjects) is large enough and correlates well with the findings in the

normal group. It would therefore seem that the curve is applicable to hyperthyroid states. The cases that fall below the normal range (twenty one female subjects, seven male subjects) are too few in number to allow any definite conclusions as to the application of the curve in hypothyroid states.

Statistical analysis of the one hundred fifty six observations on women revealed the following results. BMR readings varied from 72 per cent of normal to 162 per cent, with a mean of  $108.4$  per cent and a standard deviation of  $18.8$

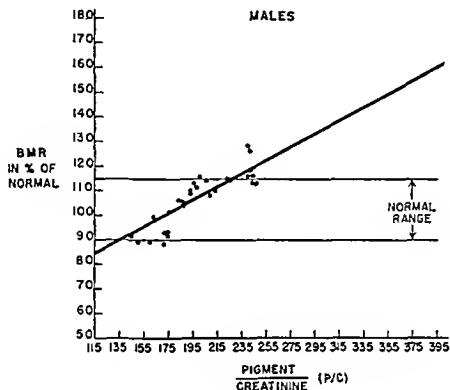


Fig. 2

per cent. Pigment/creatinine readings ranged from 124 to 536 averaging 243.8, with a standard deviation of 80.2. The coefficient of correlation, assuming a straight line relationship, was 0.92. The standard error of estimate was 7.4 per cent. The regression line is  $BMR = 54.7 + 22 \text{ pigment/creatinine}$ .

Statistical analysis upon the fifty seven observations on men revealed the following results. BMR readings varied from 83 per cent to 159 per cent averaging 109.9 per cent, with a standard deviation of 15.0 per cent. Pigment/creatinine readings ranged from 124 to 394 with a standard deviation of 52.2. The coefficient of correlation of Pearson was 0.88. The standard error of estimate was 7.1 per cent. The regression line is  $BMR = 57.0 + 25 \text{ pigment/creatinine}$ .

#### INTERPRETATION OF DATA

*Women*.—The calculation of the coefficient of correlation reveals an extremely close relationship between BMR and pigment/creatinine. The 0.92 coefficient of correlation implies a relationship close enough to allow interchangeability of the variables. The regression equation obtained is quite close to the one obtained by Ostow and Philo,<sup>4</sup> thus confirming their results. However, the higher correlation in our analysis imparts even greater reliability to the use

of the equation. Graphically, this fact is illustrated by the smaller scatter of points around our regression line.

*Men*—Ostow and Philo<sup>4</sup> published no scatter diagrams for data on men, but it can be inferred that the relationship which they discovered was too weak for practical application. However, upon applying the correlation technique to our fifty-seven determinations, we do uncover a strong relationship between the two variables. While not quite as closely related as the variables for the data on women, the coefficient of correlation (0.88) certainly indicates a strong enough relationship for the application of the regression technique. In this instance, it should be noted that our regression equation differs quite significantly from the results obtained by Ostow and Philo,<sup>4</sup> but is very close to the results obtained in the female series. Further experimental and statistical investigation would very likely reveal that the results for the male group are not significantly different from those for the female group.

#### CONCLUSION

The ratio of urinary pigment to creatinine excretion in the urine can be used as a measure of basal metabolism. The test is easily performed and is reliable.

The basal metabolic rate can be calculated from the following equations:

$$\begin{aligned}\text{Men—BMR} &= 57.0 + 25 \text{ pigment/creatinine} \\ \text{Women—BMR} &= 54.7 + 22 \text{ pigment/creatinine}\end{aligned}$$

The authors appreciate the technical assistance rendered by Miss Virginia Rehmertzer, Miss Edna Arzt, and Mrs. Jennie Shotton.

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## EXACERBATION OF ALLOXAN DIABETES IN MICE BY INJECTION OF TYPHOID VACCINE    ROLE OF THE ADRENAL GLAND

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**I**NFECTIONS as well as other forms of stress, cause the mammalian organism to increase the rate of adrenal cortex secretion. Evidence for this increased secretion in man includes (1) adrenal cortex hypertrophy,<sup>1</sup> (2) depletion of cholesterol in the adrenal cortex,<sup>2,3</sup> (3) increased urinary excretion of steroids with adrenal cortical activity,<sup>4,5,6</sup> and (4) decrease in the lymphocytes and eosinophils in the peripheral blood.<sup>7,8</sup>

Hyperscretion of the adrenal cortex, as seen in Cushing's syndrome, is associated with a diabetic syndrome which is particularly resistant to insulin. Hyperglycemia and glycosuria can be induced in both man and animals by injection of purified pituitary corticotrophic hormone.<sup>9,10,11</sup> 11 oxy corticosteroids injected into normal force fed rats have been shown to produce glycosuria.<sup>12</sup> Administration of pituitary corticotrophin,<sup>1</sup> adrenal cortical extract,<sup>13</sup> or 11 oxy corticosteroids<sup>13</sup> to diabetic rats greatly increases the glycosuria. Ingle has marshalled evidence that the glycosuria produced in rats by corticotrophic hormone or 11 oxy steroids is chiefly the result of a decreased utilization of glucose.<sup>14</sup>

Colowick, Cori, and Sleim have shown that adrenal cortical extract inhibits the hexokinase enzyme system of diabetic rat muscle but not that of normal rat muscle.<sup>14</sup> Moreover, it is well known that this hexokinase reaction is one of the main determinants of the rate of glucose utilization. These findings suggested that the exacerbation of diabetes seen during infections and trauma might be due to an increased secretion of the adrenal cortex with a resulting decrease in glucose utilization.

The following experiment was designed to investigate this possibility.

### METHODS

Following a twelve hour fast, albino mice weighing around 20 grams were given 275 mg of alloxan per kilogram of body weight, subcutaneously. Food was placed in their cages three hours after the alloxan injection. The mice which survived the alloxan injection then were divided into two groups. The mice of Group I were adrenalectomized bilaterally, ten days after their alloxan injection. Following the adrenalectomy each mouse in this group was given adrenal cortical extract (0.05 c.c. of Upjohn's Lipo Adrenal Cortex) subcutaneously, at eleven hour intervals all through the entire experiment.

Group II consisted of mice whose adrenals were left intact. These mice received 0.5 c.c. of corn oil at eleven hour intervals all through the entire experiment, corresponding to the injection of adrenal cortex extract in Group I.

Twelve days after the mice in Group I had been adrenalectomized, the mice in both groups were fasted eight hours, and a blood sugar estimation was made on a 20 c mm sample of tail blood

Two, ten, and sixteen hours after blood had been drawn for the blood sugar determination, each mouse in both groups received, intraperitoneally, 250 million killed typhoid organisms suspended in 2 c c of 10 per cent dextrose in normal saline solution Six hours after the last injection a 20 c mm tail blood sample again was drawn for a blood sugar estimation No solid food was permitted between the blood sugar determinations, but water was available as desired throughout the experiment The adrenal cortex extract (Upjohn) for Group I and the corn oil injections for Group II were continued as scheduled during the typhoid injections Each fasting blood sugar was drawn five hours after an injection of either adrenal cortical extract or corn oil

Blood sugar concentration was determined by the Somogyi procedure<sup>14</sup> The reduced copper was determined with Nelson's arsenomolybdate reagent<sup>15</sup> The final solutions were read in an Evelyn colorimeter (filter No 660) Reagent blanks and glucose standards were run with each determination Certain additional control studies, as indicated later, were also performed

In designing these experiments it was assumed that the level of the fasting blood sugar is a valid index of the severity of diabetes Mice were considered diabetic if a fasting blood sugar was over 125 mg per cent Only mice that were actually diabetic were included in the experimental Groups I and II

## RESULTS

The percentage in each group of mice that showed an increase in fasting blood glucose level after the completion of the typhoid injection is shown in Table I It can be seen that there is no significant difference between the two groups

TABLE I EFFECT OF TYPHOID VACCINE ON THE BLOOD SUGAR OF DIABETIC MICE

	NUMBER OF MICE IN GROUP	NUMBER OF MICE IN GROUP SHOWING A RISE IN FASTING BLOOD SUGAR AFTER TYPHOID VACCINE	MICE SHOWING A RISE IN FASTING BLOOD SUGAR AFTER TYPHOID VACCINE (PER CENT)
Group I (Adrenalectomized)	13	10	77
Group II (Adrenals intact)	17	13	76.5

In Table II, the milligrams per cent increase of blood sugar of each of the mice who showed a rise after typhoid is tabulated along with the average for each group

The results, when analyzed statistically, show that there is no significant difference between the increase in blood sugar of the two groups ( $P$  value = .8)

Further control studies showed the following

1 That the injections of adrenal cortical extract, in the amount we used, did not raise the blood sugar to diabetic levels in adrenalectomized nondiabetic mice (see Table III)



TABLE II RISES IN FASTING BLOOD SUGAR OF INDIVIDUAL DIABETIC MICE AFTER TYPHOID INJECTIONS

INCREASE IN MG PER CENT GROUP I (ADRENALECTOMIZED)	INCREASE IN MG PER CENT GROUP II (ADRENALS INTACT)
751	535
589	353
123	492
491	47
807	29
412	500
632	762
353	117
500	1177
85	595
	535
	74
	591
Mean 474	447
Standard Deviation $\pm 288$	$\pm 352$

TABLE III FASTING BLOOD SUGAR OF INDIVIDUAL ADRENALECTOMIZED NONDIABETIC MICE RECEIVING ADRENAL CORTICAL EXTRACT (0.05 c.c. EVERY ELEVEN HOURS)

MG %
78
70
105
102
86
Mean 88.2

2 In normal nondiabetic mice the whole experimental procedure, including typhoid injections, resulted in either no change or only a slight rise (30 mg per cent) in the fasting blood sugar. None of these normal mice exhibited the great blood sugar elevations seen in the diabetic mice receiving the typhoid injections (see Table IV).

TABLE IV CHANGES IN FASTING BLOOD SUGAR OF INDIVIDUAL NORMAL, NONDIABETIC MICE AFTER TYPHOID INJECTIONS

MG %
+13
- 8
-18
+18
+22
-27

+ Indicates an increase in fasting blood sugar

- Indicates a decrease in fasting blood sugar

3 When diabetic mice, either with or without intact adrenals were put through all the experimental procedures except for the addition of typhoid vaccine to the dextrose in saline solutions, their fasting blood sugars at the end of the experiment were not significantly greater than their initial fasting blood sugars (see Table V).

TABLE V CHANGES IN FASTING BLOOD SUGAR OF INDIVIDUAL DIABETIC MICE PUT THROUGH THE ENTIRE EXPERIMENTAL PROCEDURE EXCEPT FOR THE TYPHOID INJECTIONS

ADENALECTOMIZED MICE		MICE WITH INTACT ADRENALS	
MG	% CHANGE	MG	% CHANGE
	+30		+32
	+17		+ 4
	-32		+22
	+38		+13
	- 8		-27
			+22

+ Indicates an increase in fasting blood sugar

- Indicates a decrease in fasting blood sugar

#### DISCUSSION

The experimental findings showed that adrenalectomized mice receiving a fixed daily amount of adrenal cortex extract exhibited as great a rise in fasting blood sugar after a course of typhoid injections as mice with intact adrenals. Since these adrenalectomized mice were incapable of increasing their own adrenal hormone secretion, it is concluded that adrenal hypersecretion (either adrenal cortex or medulla) was probably not the cause of the elevation of blood sugar produced by typhoid vaccine injections in these diabetic mice.

It is possible that accessory adrenal tissue had begun to function in these adrenalectomized mice, but they began receiving adrenal cortex extract immediately following adrenalectomy and hence had little stimulus for the growth of accessory adrenal tissue. Following the experiment, no more adrenal cortex extract was given, and all of the adrenalectomized mice died in a few days. If many had had significant amounts of accessory adrenal tissue, one would have expected more survivors.

If one is justified in applying such experiments to the problem of infections in diabetic patients, it would appear that increased adrenal activity is not the cause of the aggravation of clinical diabetes by infections.

It may be justifiable to speculate on other possible explanations of the exacerbation of diabetes with infections.

It is possible that the anterior pituitary hormone which inhibits the hexokinase reaction<sup>17</sup> is secreted in increased quantities during infections and stress, and that the hypersecretion of this hormone causes decreased glucose utilization and hence an exacerbation of diabetes. This possibility is supported by the observation that the anterior pituitary gland secretes an increased amount of another hormone, the corticotrophic hormone, in response to stress.<sup>2</sup>

Kun<sup>18</sup> has recently reported that meningococcal endotoxin causes *in vitro* a decrease in glucose utilization by rat muscle extracts, probably by inhibiting the hexokinase system. This inhibition of glucose utilization did not occur if Zn-free insulin was added to the reaction mixture. It may be that the endotoxins of various other types of bacteria cause an inhibition of the hexokinase system, with a resulting decrease of glucose utilization.

In diabetes mellitus, glucose utilization for a given level of blood sugar is considerably decreased, and any mechanisms, such as the two mentioned, which

further impair glucose utilization will cause a marked exacerbation of the manifestations of diabetes, such as hyperglycemia, glycosuria, and excessive mobilization of fat for energy metabolism.

#### SUMMARY

The exacerbation of diabetes in mice following typhoid injections is not caused by an increased secretion of either medullary or cortical hormones of the adrenal gland. If one is justified in applying this finding to comparable clinical situations, the exacerbation of clinical diabetes during infections probably also is not caused by adrenal hypersecretion.

We wish to thank Dr. E. Gifford Upjohn, of the Upjohn Company, Kalamazoo, Mich., for generously supplying us with Lipo-Adrenal Cortex (Upjohn).

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## HYPERGLYCEMIA AND GLUCOSURIA FOLLOWING THYROID ADMINISTRATION IN ALLOXAN TREATED RATS

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SHPLEY and Rannefeld<sup>1</sup> showed that the glucose tolerances of rats which received small repeated doses of alloxan were similar to those of diabetic animals. Histologic study of islet tissue in rats injected intravenously every other day four times with 20 mg per kilogram doses of alloxan revealed apparently intact islets of Langerhans with the methods used.<sup>2</sup> Glucose tolerances in these animals were decreased. It was concluded that functional impairment of the beta cells may be caused by small repeated doses of alloxan.

Animals with islet tissue functionally altered by alloxan were fed thyroid in this experiment to determine if histologic changes would take place. One group of rats was adrenalectomized to determine the influence of lowered blood sugar level on islet tissue in alloxanized-thyroid fed animals.

### MATERIALS AND METHODS

Four groups of white albino rats of the same stock and age, weighing about 100 grams, and of mixed sex were used. Animals in the first three groups received four intravenous injections of alloxan (20 mg per kilogram), one every other day. Group 4 animals were controls and did not receive alloxan. Fasting blood sugars in all animals were within 80 to 120 mg per cent using a modified Folin Wu procedure.<sup>1</sup> Glucose tolerances determined ten days after the last injection of alloxan were impaired in the first three groups. Group 4 had normal glucose tolerances. Glucose tolerance tests were conducted by withdrawing a sixteen hour fasting blood sample, injecting 0.5 Gm per kilogram glucose intravenously in 20 per cent solution, and drawing blood samples at one half, one, and two hour intervals. Pancreatic biopsy revealed normal islet tissue with the methods used. Animals in Group 3 were adrenalectomized and given 1 per cent saline to drink. Animals were then tube fed powdered thyroid (Armour), 0.1 Gm per kilogram in water, daily for one month. Body weights of the animals were followed. All animals were allowed to eat ad libitum a prepared dog biscuit.

Following this treatment, twenty four hour urine specimens were examined for glucose.<sup>†</sup> Animals were placed in metabolism cages for forty eight hours and tube fed three times a day. Served a diet containing 20 per cent protein, 76 per cent carbohydrate, 4 per cent fat, adequate salts and vitamins. Each cubic centimeter of diet contained 182 large calories per milliliter. This diet was a modified one of a type suggested by Dr. R. Reinecke, Department of Physiology. Glucose tolerances were also repeated. These procedures were done three weeks after the last dose of thyroid. All animals were sacrificed without starvation, at the same time, one month after the last thyroid feeding, and the pancreases were placed in Bouin's fixative. The tissue was sectioned at 4 microns. After staining with Heidenhain's iron hematoxylin, every tenth section was examined and the number of alpha and beta cells counted and the size of the islet noted.

From the Department of Anatomy, University of Minnesota Medical School.

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\*Senior Research Fellow, United States Public Health Service.

†Alloxan monohydrate, Eastman Kodak Company.

‡Quantitative Benedict's method.

## RESULTS

Glucosuria and fasting hyperglycemia occurred in animals that were fed thyroid following four small repeated doses of alloxan (Tables I, II, and III). These animals were observed for a month after termination of thyroid administration and the glucosuria remained. Microscopic examination of islets in these animals revealed a reduction in the number of beta cells (Table IV). In animals that received alloxan only and were followed for a comparable length of time, no spontaneous hyperglycemia occurred (Tables I and II). Glucose tolerance curves of normal rats, four dose alloxan animals (20 mg per kilogram), and four dose alloxan animals which were fed thyroid in addition (0.1 Gm per kilogram) are shown in Fig. 1. Islets in animals which received alloxan only appeared normal. Similarly, in animals which received only thyroid, no hyperglycemia, glucosuria, nor histologic alteration of the islets appeared with the methods used (Tables I and II).

In the group that was adrenalectomized following alloxan and then fed thyroid, no clinical diabetes developed (Tables I, II, and III), nor were any changes in the islets noted. It would appear that a lowering of blood sugar might have been the protective mechanism in this group.

In the alloxanized thyroid fed animals which developed clinical diabetes, it would seem that the initial functional impairment of the beta cells by alloxan was essential if thyroid feeding produced hyperglycemia, glucosuria, and reduction in the number of beta cells.

Since the beta cells are centrally located within the rat islet, alpha cells being present only at the periphery, evaluation of beta versus alpha cell damage is unequivocal. The beta cells of the rat are larger than the alpha cells, and on the basis of size, position, and affinity of the beta cell granules for hematoxylin, counts of the cell types can be made readily on thin sections stained properly with iron hematoxylin and eosin. Identification of cell types was checked against Bouin fixed material stained by the method of Gomori.<sup>2</sup> In the animals fed thyroid following the four small doses of alloxan, the islets were smaller (fewer beta cells) and the alpha cells were not confined to the periphery as in the case of islets from either untreated rats or animals which received only the four small doses of alloxan, or were fed thyroid with no other treatment, or were fed thyroid but were adrenalectomized following alloxan.

TABLE I. HYPERGLYCEMIA AND GLUCOSURIA FOLLOWING ALLOXAN AND THYROID

GROUP	NUMBER OF ANIMALS	TREATMENT	RESULTS
1	11	{ Alloxan Thyroid	{ Fasting hyperglycemia Glucosuria Reduced number of beta cells
2	6	Thyroid	{ Normal fasting blood sugar No glucosuria No reduction in beta cells
3	4	{ Alloxan Adrenalectomy Thyroid	{ Fasting blood sugar 60-80 mg % No glucosuria No reduction in beta cells
4	4	Alloxan	{ Normal fasting blood sugar No glucosuria No reduction in beta cells

## HYPERGLYCEMIA AND GLUCOSURIA FOLLOWING THYROID ADMINISTRATION IN ALLOXAN TREATED RATS

DAVID W. MOLANDER, M.S., M.D.,\* AND ARTHUR KIRSCHBAUM, M.D., PH.D.  
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SHPLEY and Rannefeld<sup>1</sup> showed that the glucose tolerances of rats which received small repeated doses of alloxan were similar to those of diabetic animals. Histologic study of islet tissue in rats injected intravenously every other day four times with 20 mg per kilogram doses of alloxan revealed apparently intact islets of Langerhans with the methods used.<sup>3</sup> Glucose tolerances in these animals were decreased. It was concluded that functional impairment of the beta cells may be caused by small repeated doses of alloxan.

Animals with islet tissue functionally altered by alloxan were fed thyroid in this experiment to determine if histologic changes would take place. One group of rats was adrenalectomized to determine the influence of lowered blood sugar level on islet tissue in alloxanized-thyroid fed animals.

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Four groups of white albino rats of the same stock and age, weighing about 100 grams, and of mixed sex were used. Animals in the first three groups received four intravenous injections of alloxan (20 mg per kilogram), one every other day. Group 4 animals were controls and did not receive alloxan. Fasting blood sugars in all animals were within 80 to 120 mg per cent using a modified Folin Wu procedure.<sup>1</sup> Glucose tolerances determined ten days after the last injection of alloxan were impaired in the first three groups. Group 4 had normal glucose tolerances. Glucose tolerance tests were conducted by withdrawing a sixteen hour fasting blood sample, injecting 0.5 Gm per kilogram glucose intravenously in 20 per cent solution, and drawing blood samples at one half, one, and two hour intervals. Pancreatic biopsy revealed normal islet tissue with the methods used. Animals in Group 3 were adrenalectomized and given 1 per cent saline to drink. Animals were then tube fed powdered thyroid (Armour), 0.1 Gm per kilogram in water, daily for one month. Body weights of the animals were followed. All animals were allowed to eat ad libitum a prepared dog biscuit.

Following this treatment, twenty four hour urine specimens were examined for glucose.<sup>†</sup> Animals were placed in metabolism cages for forty eight hours and tube fed three times a day 8 c.c. of a diet containing 20 per cent protein, 76 per cent carbohydrate, 4 per cent fat, adequate salts and vitamins. Each cubic centimeter of diet contained 182 large calories per milliliter. This diet was a modified one of a type suggested by Dr. R. Remecke, Department of Physiology. Glucose tolerances were also repeated. The same procedures were done three weeks after the last dose of thyroid. All animals were sacrificed without starvation, at the same time, one month after the last thyroid feeding, and the pancreases were placed in Bouin's fixative. The tissue was sectioned at 4 microns. After staining with Heidenhain's iron hematoxylin, every tenth section was examined and the number of alpha and beta cells counted and the size of the islet noted.

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TABLE III TWENTY FOUR HOUR GLUCOSE EXCRETIONS OF RATS WHICH RECEIVED FOUR INTRAVENOUS INJECTIONS OF ALLOXAN (20 MG/KG) FOLLOWED BY THYROID (0.1 GM/KG) DAILY FOR ONE MONTH

Animals	2	3	4	5	6	9	10	12
Gm per kg per 24 hr	4	14	26	20	10	24	18	16

TABLE IV PERCENTAGE OF ALPHA AND BETA CELLS PRESENT IN ISLETS OF RATS THAT RECEIVED FOUR INTRAVENOUS INJECTIONS OF ALLOXAN (20 MG/KG) AND THYROID DAILY FOR ONE MONTH (0.1 GM/KG) COMPARED WITH THAT PRESENT IN ANIMALS THAT RECEIVED ONLY ALLOXAN ONLY THYROID AND THYROID AFTER ADRENALECTOMY FOLLOWING ALLOXAN

GROUP	PERCENTAGE DISTRIBUTION	
	ALPHA CELLS	BETA CELLS
{ Alloxan	70	30
{ Thyroid	90	10
{ Alloxan	50	50
{ Thyroid	50	50
{ Alloxan	50	50
{ Adrenalectomy	50	50
{ Thyroid		

## COMPARATIVE GROWTH CURVES

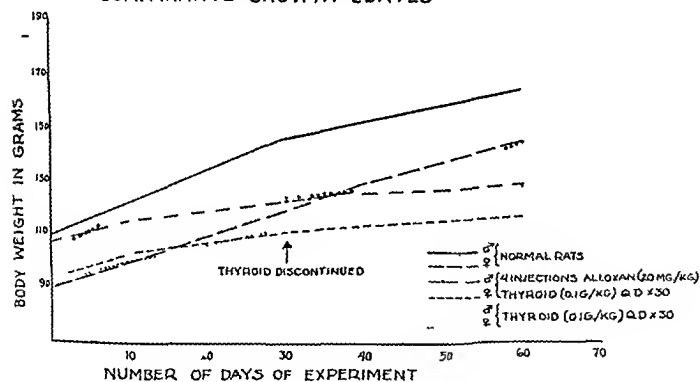


Fig 2

Thyroid effect on body weight is seen in the comparative growth curves (Fig 2). Animals which received only alloxan in small amounts gained in body weight at a rate similar to normal animals, however, the group that received thyroid in addition to alloxan was retarded (Fig 2). Animals which received only thyroid for a month were transiently stunted in growth.

## SUMMARY

Rats given four small doses of alloxan (20 mg per kilogram) and then red thyroid daily for one month (0.1 Gm per kilogram) developed hyperglycemia

and glucosuria. Islets in these animals showed a reduction in the number of beta cells. Retarded growth occurred.

Animals given alloxan but no thyroid and followed a similar length of time showed neither hyperglycemia nor glucosuria, and islet tissue appeared intact with the methods used. Growth retardation was not noted.

Animals which received only thyroid for a similar period were retarded in growth, showed neither hyperglycemia nor glucosuria, and had normal-appearing islet tissue.

The small group that was adrenalectomized following alloxan injection and then fed thyroid did not develop hyperglycemia, glucosuria, nor show any islet changes. Lower blood sugar level (reduced gluconeogenesis) following adrenalectomy may have been a factor in inhibiting beta cell damage.

By functionally altering the beta cells with small repeated doses of alloxan and then feeding thyroid it was possible to detect the added metabolic strain of thyroid on islet tissue.

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## THE EXCRETION OF PENICILLIN IN HUMAN MILK

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THE extensive literature dealing with the concentrations of penicillin in body tissues and fluids following intramuscular administration contains few references to the fate of penicillin in human milk. Seeley and co-workers<sup>1</sup> found no penicillin in bovine milk after injecting 500,000 units. Greene and associates found a concentration of 0.015 to 0.06 unit per cubic centimeter in human milk after a single intramuscular injection of 100,000 units of penicillin. In view of the scarcity of the data a study of the excretion of penicillin in human milk was undertaken.

### MATERIALS AND METHODS

The concentration of penicillin was determined by the serial dilution method of Kolmer.<sup>2</sup> Each series consisted of six tubes with a final dilution of 1:32. One tenth of a cubic centimeter of a 1:1,000 dilution of a twenty-four hour bouillon culture of *Staphylococcus aureus* (Heathley strain) was added to 1 cc. of the medium.

Since the opacity of milk precluded observations on turbidity we estimated growth inhibition with the aid of color indicators. Whereas methylene blue is suitable for the estimation of penicillin concentration in bovine milk, since the reduction of the dye and the coagulation of the milk yield a clear end point in the case of human milk we found bromthymol blue to be a more suitable indicator. Human milk normally has a pH of 7.1 to 7.6. Bromthymol blue changes color from blue to green at pH 0.6, and turns yellowish at pH 0.0.

The indicator was made up as follows: Bromthymol blue, 1.0 Gm. NaOH N/10 25 cc., aqua destillata, 475 cc. 12 cc. of indicator per 1 liter of medium.

Whenever possible, the dilutions were made up with milk from the same donor. In a few cases when only a small quantity of milk was obtainable dilutions were made up with milk from another woman. Milk was taken before the injection of penicillin and was boiled for five minutes to ensure sterility. It was found that a 1:10 dilution of milk in normal saline could replace whole milk as a satisfactory diluent, without inhibiting the growth of the test organism. In consequence of this observation both whole milk and milk saline were regularly employed as diluents in parallel series.

We observed that when we added a few drops of 0.1N NaOH to the medium, we obtained clearer color distinction. The slight alkalization enhances the blue color and at the same time promotes the growth of the staphylococcus. To test the reliability of the color changes, we inoculated agar slants from the dilution series and observed that all dilutions that had not turned green had remained sterile. Readings were made after twenty-four hour incubation at 37°C. The penicillin sensitivity of the test strain was examined with each experiment. The strain was sensitive to 0.03 unit per cubic centimeter and yielded the corresponding color changes. Milk in itself does not destroy the antibiotic activity of penicillin in vitro since we found that solutions of penicillin in milk had the same antibiotic action as did corresponding solutions in normal saline.

In a preliminary experiment to determine whether penicillin is excreted in milk, we examined the milk of a bitch weighing 18 kilograms three days after whelping. A single intramuscular injection of 100,000 units diluted in 2 cc. normal saline was given in the thigh. Milk was taken before the injection and at intervals of one, two and four hours after the injection. The sample taken before penicillin injection had no antibiotic activity,

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whereas varying amounts of penicillin appeared following the injection (0.12 unit per cubic centimeter after one hour, and 0.24 unit per cubic centimeter after two and four hours)

We studied penicillin concentration in human milk in the following manner. A single intramuscular injection of 200,000 to 600,000 units of penicillin (crystalline G, potassium salt) dissolved in normal saline was administered to thirteen women two to eight days after delivery. The injection was given as soon as the infant was taken from the breast. In one instance, four additional injections of 30,000 units each were given at three hour intervals before and after the main injection of 400,000 units. Milk samples for the determination of penicillin concentrations were taken before the injection and one, two, four, and six hours after the injection. On three occasions, samples of milk taken nine hours after the injection were also examined. All samples were taken from the same breast. Before withdrawing the milk, the nipple and surrounding area were cleaned with iodine, washed with alcohol, and dried with a sterile towel. For purposes of comparison a number of blood samples were also taken two and six hours following injection. In each case sterility tests of the milk were done, and contaminated samples were discarded. Control experiments were regularly carried out to prove that the milk taken before the injection of penicillin possessed no antibiotic properties.

### RESULTS AND DISCUSSION

The results of the study are summarized in Table I.

TABLE I. CONCENTRATION OF PENICILLIN (U/C C) IN MILK AND SERUM FOLLOWING INTRAMUSCULAR ADMINISTRATION

CASE SERIAL NO	DAYS AFTER DELIVERY	DOSAGE (UNITS)	HOURS AFTER INJECTION							REMARKS
			1	2		4	6		9	
			MILK	MILK	SERUM	MILK	MILK	SERUM	MILK	
1	2	200,000	0.06	0.06	-	-	-	-	-	
2	5	200,000	≥ 0.06	≥ 0.06	≥ 0.96	-	≥ 0.06	< 0.03	-	
3	5	200,000	0.03	≥ 0.06	0.96	≥ 0.06	≥ 0.06	< 0.03	-	
4	6	200,000	0.36	-	-	0.48	-	-	-	
5	3	300,000	< 0.03	< 0.03	0.96	< 0.03	< 0.03	-	< 0.03	
6	2	400,000	0.03	0.06	0.72	-	≥ 0.12	< 0.03	-	
7	6	500,000	0.06	0.12	0.72	0.12	0.12	< 0.03	-	
8	4	500,000	0.12	0.24	-	-	0.24	0.12	0.12	
9	6	500,000	0.12	0.24	1.92	0.24	0.12	< 0.06	0.06	
10	8	500,000	0.24	≥ 0.96	≥ 0.96	≥ 0.96	0.24	-	-	
11	2	600,000	0.12	-	-	0.24	0.24	-	-	
12	3	600,000	-	0.24	-	0.36	0.24	0.48	-	
13	4	400,000	0.48	0.48	-	0.48	≥ 0.48	-	-	Eight additional injections of 30,000 U each every 3 hr

≥ More or equal < less than — not examined

As may be seen from the table, significant quantities of penicillin were found in the milk following intramuscular injection, in all but one of the cases studied (Case 5). Within one hour after injection, penicillin was found in appreciable quantities, the maximum concentration observed having been 0.36 unit per cubic centimeter. Two hours after the injection, the level was in most cases twice as high as after one hour, and it was still rising steadily at four hours. This rise in concentration was observed in almost every instance. The penicillin concentration began to fall six hours after injection, but remained as high as the one-hour level, or higher. Nine hours after injection, penicillin was still present in the milk of two of the three patients examined at this time. In Case 13, in which the patient had received injections of 30,000 units every three

hours, in addition to the main injection of 400 000 units, the level was maintained at 0.48 unit per cubic centimeter throughout six hours. In Case 5 although the dose injected was large, and although the penicillin level in the serum was high (0.96 unit per cubic centimeter), no penicillin could be detected in the milk.

## Average Values Of Penicillin Levels In Human Milk

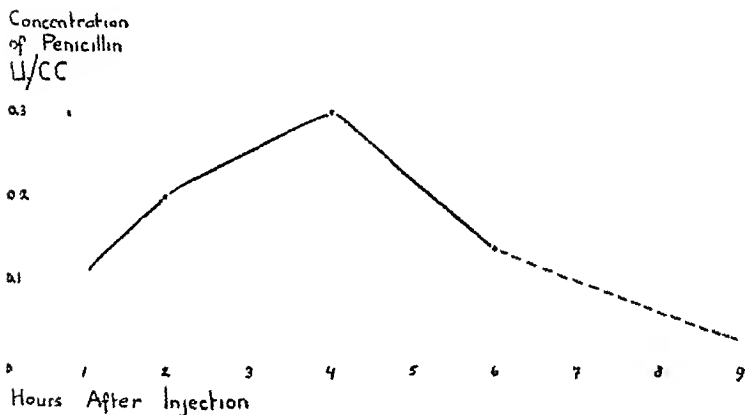


Fig 1

The steady increase and persistence of penicillin in human milk during the first four hours after intramuscular injection, followed by subsequent gradual decrease, is clearly demonstrated in Fig 1.

As for the relationship between the levels of penicillin in serum and milk we found that at the end of two hours the concentration of penicillin in the milk was 10 to 15 per cent of that in the serum. Subsequently the milk level remains constant or rises, while the blood level falls. In milk as in serum, no close relationship exists between the dose of penicillin injected and the level obtained, but in general higher penicillin levels were observed after larger doses. The results of this study explain the beneficial therapeutic effects of intramuscularly injected penicillin on mastitis. The penicillin sensitivity of most penicillin susceptible strains of bacteria encountered in this country, as examined by Gurevitch,<sup>4</sup> lies within the range of the levels we have demonstrated in milk.

Whether suckling milk containing penicillin can favorably affect oral infections in the infant, caused by organisms sensitive to penicillin, is a question requiring investigation.

## SUMMARY

A study was made on the penicillin concentration in milk of thirteen women after intramuscular administration of the drug. Significant quantities of penicillin were found in the milk of twelve of these subjects.

Penicillin was found in milk one hour after the injection. Up to four hours after the injection the level rose, followed by a gradual fall in the concentration.

In two of three subjects examined, penicillin was still found nine hours after the injection.

We are indebted to Dr. N. Grossowicz for his helpful advice.

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## BACTERIMETRIC STUDIES III BLOOD LEVEL STUDIES ON TEROPTERIN METABOLISM

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THIS work was undertaken because of the evident potentialities of teropterin in cancer therapy.<sup>1</sup> The approach is based on two premises: (a) the observed clinical effects of teropterin treatment seem to cover the whole range from apparent ineffectiveness, and possibly stimulatory tendencies, to genuine regressions of malignant growths, and (b) in view of what is known about individual variations in vitamin metabolism it seems possible that individual differences in the metabolic handling of teropterin play an important role in determining its clinical effectiveness.

Because of available evidence indicating rapid metabolic liberation of folic acid (folic acid, pteroylglutamic acid) from teropterin (pteroylglutamic acid) and because of the presumptive procarcinogenic activity of the former,<sup>2</sup> it seemed desirable to determine the blood concentrations of these two compounds resulting from administration of teropterin.

### METHOD

A simplified analytic method has been developed for this work which is based on the well established differences<sup>4</sup> in the growth responses of *Lactobacillus casei* 7169 and *Streptococcus faecalis* 8043 (*Str. lactis* R.) to the two compounds. The experimental procedure is briefly as follows:

Blood samples of approximately 0.015 cc are taken from the finger tip in a Thoma leucocyte diluting pipette and after dilution with 20 volumes of water are placed in capped vials and stored in a freezing compartment until they are analyzed.

For the bacterimetric determinations a single basal medium of low folic acid content is used for both organisms. This medium is based on the *L. casei* medium of Tepley and Elvehjem,<sup>5</sup> differing from the latter in that vitamins (using pyridoxamine) and phosphates are present according to the specifications of Roberts and Snell,<sup>6</sup> and in that casein hydrolysate and peptone are replaced by vitamin free enzymatic casein hydrolysate<sup>7</sup> in an amount of 0.12 cc per cubic centimeter of finished medium. Culture tubes and the procedures of sterilization, inoculation, incubation and photoelectric turbidity measurement are those described by us elsewhere,<sup>7</sup> except that readings were taken at 675 m $\mu$  and the tubes contained only 6 cc of solution. This volume is sufficient if the tubes are slightly raised in the Coleman cuvette carrier by means of a fixed support.

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Nutritional Biochemicals Corporation Cleveland Ohio

For purposes of convenience in measuring, calculating, and recording, the one trillionth part of a mol ( $1 \times 10^{-12}$  mol), called briefly 1 trillmol and abbreviated 1 tM, has been adopted as a reference unit for the present work with the pteroyl compounds. It corresponds to 0.441 millimicrogram of pteroylglutamic acid or 0.699 millimicrogram of pteroyltriglutamic acid.

Inocula of the two organisms are grown in a medium which contains, per liter, 20 Gm Bacto-casitone, 5 Gm Bacto-peptone, 20 Gm anhydrous sodium acetate, 20 Gm glucose, 2.5 Gm each of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ , 200 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 10 mg each of  $\text{NaCl}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ . If this medium is inoculated from a culture of either organism freshly grown in the same medium, active inoculum cultures are obtained after approximately sixteen hours at 37 to 38°. The cells are washed once with water and diluted to an O.D. of 100.

The standard series consist of single tubes containing 0, 0.15, 0.30, 0.45, 0.60, 0.90, 1.20, and 1.50 tM folacin for *L. casei* and twice these quantities for *St. faecalis*. Determinations performed under these conditions indicate a ratio of approximately 1 for the molar activities of folacin and teropterin for *L. casei*, and for *St. faecalis* an activity of teropterin of approximately 2 per cent of that of folacin. In the actual determinations only folacin standards, for both organisms, have been run and, as a practical approximation, the *L. casei* results of the unknown have been considered as the measure of the sum of folacin and teropterin, and the *St. faecalis* results as the measure of folacin.

For analysis the original 1:21 blood dilutions are treated as follows. A 0.10 c.c. portion is further diluted with 9.4 c.c. of water. In order to minimize subsequent interference of turbidities arising from blood proteins, these dilutions are autoclaved at 120° for two and one-half minutes and, after cooling, centrifuged. For each blood specimen four assay tubes are then set up, containing 0.5 and 1.0 c.c. of the 1:2,000 dilutions for *L. casei*, and 1.5 and 3.0 c.c. for *St. faecalis*. Volumes are completed to 6 c.c. with inclusion of 3 c.c. of the basal medium. Because in some cases small residual turbidities remain after the preliminary autoclaving and centrifugation, initial optical density readings are taken on all tubes after the final sterilization. These readings are subtracted as blanks from the subsequent readings, taken after sixteen and twenty-two hours of incubation of the inoculated tubes at 37.7°. The practice of taking readings at two time levels, as well as two quantity levels, was adopted as an additional means of guarding against possible errors arising from unknown growth factors.

## RESULTS

The results obtained so far are summarized in the seventeen charts of Fig. 1 (1 to 15) and Fig. 2 (16 and 17). The full lines show the blood concentrations found for folacin and the broken lines show the concentrations of teropterin. The units of the ordinate, which in all instances are drawn to the same scale, are trillimols per cubic centimeter of blood. The abscissa units are hours. Except for charts 13, 16, and 17, then scaling is also uniform. The

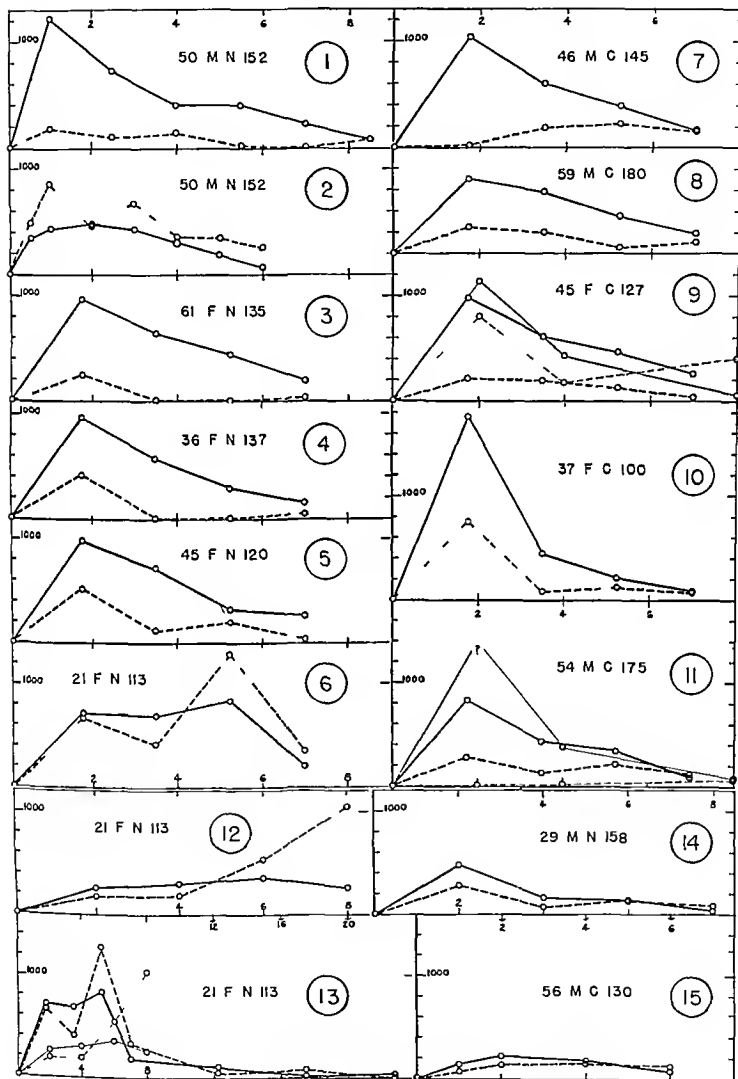


Fig 1—Results of teropterin administration as explained in text. Administration at 0 time.

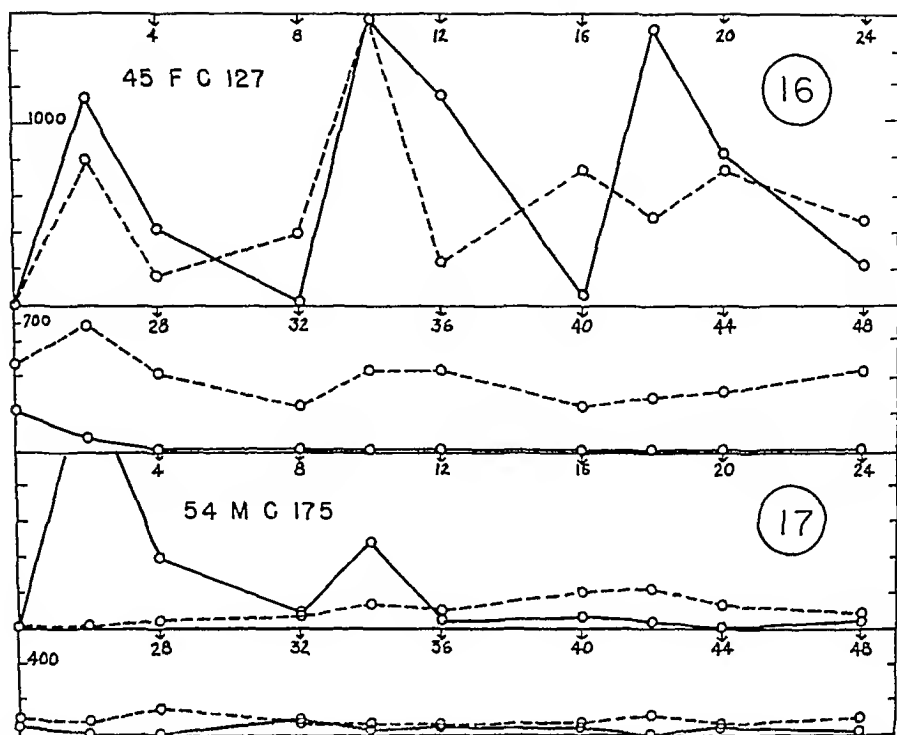


Fig 2—Results of teropterin administration as explained in text. Administrations at 0-8 and 16-hour period

numbers-and-letters code shown above each graph characterizes the subject of the experiment, in the following manner: age, sex (M or F), normal (N) or cancerous (C), weight. Thus, 50 M N 152 indicates a normal man of age 50 weighing 152 pounds. The initial concentration for both substances is in all cases shown as zero. The actual normal levels found range from about 5 to 50 tM for folacin. Although as a rule no teropterin was found, a few subjects showed a low initial teropterin activity. Pending further investigation of these observations, the initial levels have been ignored, since in view of their low values, compared with those resulting after administration of Teropterin, no serious error is introduced thereby.

The injections were made intramuscularly in the upper arm. Blood samples were taken from the fingers of the opposite arm. For the 20 mg administration the dry preparation was used, dissolved in 1 cc of saline for the injections. For the 10 mg injections the solution commercially available in vials was used.

Chart 1 shows the results of oral and chart 2 the results of intramuscular administration of 20 mg teropterin in the same subject. Because the latter method yielded much higher teropterin levels, further studies by the oral route so far have not been made. Charts 3 to 6 show the results of 20 mg injections in four additional normal subjects. Charts 7 to 11 show results obtained in five patients with cancer with 20 mg injections. The thin lines in charts 9 and 11



indicate data obtained in later experiments with the same subjects (see charts 16 and 17). Charts 12, 11, and 15 describe the results of 10 mg injections on two normal subjects and one patient with cancer. The experiments described in charts 6 and 12 were done on the same subject. Because of the unusual nature of the delayed response encountered in the experiment of chart 12 (which preceded that of chart 6) blood sampling was extended until twenty-two hours after injection in the experiment of chart 6. Both experiments are plotted together in chart 13.

Charts 16 and 17 describe for two patients with cancer who had been subjects of previous experiments (charts 9 and 11 respectively), the results of an experiment extending over forty-eight hours. During the first twenty-four hours three teropterin injections of 20 mg each were given at eight-hour intervals (zero, eight and sixteen hours).

*Precision*—Prior to further discussion the precision of the data shown in Figs. 1 and 2 should be indicated. A statistical summary of relevant results is given in Table I.

Each entry in this table represents an average obtained from approximately 130 pairs of readings. As stated each blood specimen was analyzed in two concentrations and each of the resulting individual tubes was read after two periods of growth. The resulting "quantity pairs" were always in a 1:2 ratio, and the "hour pairs" consist of readings taken after sixteen and twenty-two hours of growth. During this period the standards showed an average increase in optical density of about 40 per cent. The figures given in Table I represent millimols per cubic centimeter of blood, i.e. the same units as are used in Figs. 1 and 2. Average drift means, for the hour pairs, the

TABLE I

	HOUR PAIRS		QUANTITY PAIRS	
	AV. DIFFERENCE (tM)	AV. DRIFT (tM)	AV. DIFFERENCE (tM)	AV. DRIFT (tM)
<i>L. casei</i>	70	-20	90	+30
<i>Str. faecalis</i>	50	+10	50	+20

net difference between the averages of all twenty-two hour and all sixteen hour readings, and for the quantity pairs, the net difference between the averages resulting from all double and all single levels. Thus the table shows that in the *L. casei* measurements the final figures resulting from the sixteen hour and the twenty-two hour readings differ in the average by 70 tM and the value resulting from the later reading is, in the average, 20 tM lower than the result of the earlier reading. Further the results of the single and double quantity samples differ in the average by 90 tM and the value resulting from the larger sample is, in the average, 30 tM higher than the result of the smaller sample. Since the folacin values derive directly from the *Str. faecalis* readings while the teropterin values result from the difference between the *L. casei* and the *Str. faecalis* readings their error would in the average be somewhat larger than that of the folacin values. It is concluded from the statistical evidence that

by combining the results of the hour pairs and the quantity pairs into one single average, as has been done in Figs 1 and 2, a reasonable compensation of errors is obtained and that the procedure as used is adequate to reveal the major trends in the blood picture resulting from administration of teropterin.

*Accessory Observations*—A few additional observations made in the course of this work warrant only brief mentioning. Any effects which meals may have on the folacin-teropterin picture of the blood appeared to be negligible in terms of the concentration levels resulting from administration of the pure compound—Quantitative experiments showed that no significant changes result from refrigerator storage of the primary, 1:21, blood dilution—A preliminary study of normal folacin levels in the blood was made, involving six normal persons and six patients with cancer. The following results, in terms of millimols per cubic centimeter of blood, were obtained: 5, 9, 9, 15, 17, and 28, for the normal subjects, and 8, 9, 15, 25, 28, and 35 for the patients with cancer. In the same series the recovery of folacin added to the blood samples was also studied. While the evidence obtained indicates absence of major secondary effects of the blood in the folacin determinations, a more careful study of this angle seems desirable. Our values of 5 to 35 (median 17) mM per cubic centimeter of whole blood, obtained without any treatment except aqueous hemolysis, may be compared with the values obtained by Schweigert and Pearson<sup>9</sup>: 1 to 3 mM on oxalated blood without taka diastase treatment and an average of 86 mM on blood incubated with taka diastase. Denko and co-workers<sup>10</sup> obtained a range of 27 to 120 mM after taka diastase treatment. This comparison suggests that under our conditions some but not all of the bound folacin is set free.

#### DISCUSSION

The dominating effect of the administration of teropterin which emerges from the present observations is the rapid appearance of folacin in the blood stream. The folacin concentration appears to attain its maximum approximately two hours after injection. If the total circulatory distribution volume of the body is assumed to be 21 to 25 per cent of the body weight,<sup>8</sup> the peak values for the sum of teropterin and folacin in the individual subjects average 62 or 74 per cent, respectively, of the theoretical concentration. At the observed peak periods (which may not be the precise actual peak periods) the relative shares of folacin and teropterin are, in the average, 71 and 29 per cent of the total. Six hours after injection an average of 27 per cent of the folacin present at the peak period is left, while the teropterin level at this time has declined to an average of 36 per cent of its value at the peak period. In the calculation of these averages all injection experiments, except those shown in charts 6 and 12, have been used. These will be discussed below.

In terms of the theoretical maximum, based on a distribution volume of 25 per cent of the body weight, the percentage of the injected teropterin which six hours after injection is still accountable in the circulation, as teropterin or folacin, averages 22. On the basis of the urinary excretion

studies of Jukes Franklin Stokstad, and Bochner one may anticipate a six hour folacin excretion of 60 or 70 per cent of the total dose after administration of 20 mg of teropterin. Thus within a margin of 10 or 20 per cent the total dose seems fairly well accounted for between excretion and excretion.

These are average figures. As is evident from inspection of Figs 1 and 2 among the individual subjects the rise and decline of folacin concentration is relatively uniform in character in comparison with the great individual differences encountered among the teropterin patterns. While folacin shows in practically all cases a pattern of steady decline after the initial rise the primary decline following the postinjection peak of teropterin is in many cases followed by a secondary surge. Because of its occurrence in three quarters of the cases considered, the secondary surge is thought to be more than a fortuitous result of combined errors. Furthermore, at the end of the short term experiments (up to eight hours) the teropterin trend is upward in one third of the cases while the folacin trend is always downward. The two forty eight hour studies (charts 16 and 17) show in further confirmation of the different character of the teropterin and folacin patterns that the teropterin to folacin ratio tends to change from a value of less than 1, which is characteristic for the early postinjection periods, to a ratio larger than 1 which is maintained for a considerable period after the last injection.

Because there are equally good reasons for considering folacin an adjuvant of malignant growth as there are for considering teropterin an antagonist, one may theorize that the teropterin to folacin ratio resulting from teropterin administration is more important than the size or frequency of dosage per se. Accordingly the conditions governing establishment and maintenance of a favorable ratio should be studied. The obvious differences in the rates of disappearance of the two compounds suggest a slower rate of excretion of teropterin and/or a process of storage or binding from which teropterin is gradually released.

From a practical point of view it would seem important to know if the response pattern of a subject is determined by stable individual metabolic characteristics or whether the response of the same individual varies substantially and unpredictably from day to day. Among the present data there are a few bearing on this question. Charts 9 and 11 show two cases in which a second analysis was carried out on the same person after an interval of two weeks. In either case the second picture differs substantially from the first. Charts 6 and 12 show two experiments performed on another subject. In this case the dosage levels differed and the interval was six days. The response picture of this person is particularly interesting because of the unusual delay in the attainment of the top levels for both folacin and teropterin but particularly the latter. As chart 13 shows in which the data of charts 6 and 12 are combined this characteristic is in evidence in both experiments despite other differences.

## SUMMARY

Studies, using *L casei* and *Str faecalis*, of the fate of pteroylglutamic acid in human subjects indicated that two hours after intramuscular injection approximately two-thirds of the dose (20 mg) were present in the circulation. Of this total about two-thirds were present as the mono- and one-third as the triglutamate. Subsequently the concentration of the former seemed to decline more rapidly than that of the latter. Different individuals show considerable variations from the average metabolic pattern.

## ADDENDUM

Upon completion of this article a paper by Schweigert on metabolic observations after administration of pteroylglutamic acid and pteroyltriglutamic acid appeared<sup>11</sup>. Despite differences in approach and methods, the findings of this work are similar to some of our results. In both investigations maximum blood levels were observed approximately two hours after injection, although the route was intramuscular in one case and intravenous in the other. However, in our study the maximum value obtained after two hours (average, about 1,400 tM, average weight of subject, 140 pounds) was more than twice as high as that of Schweigert, although the injected quantity of the triglutamic acid was essentially the same. As noted, our normal folic acid levels are also higher than those recorded by Schweigert. His tentative conclusion "that some of the injected triglutamate was not cleaved for as long as four to eight hours after injection" is fully confirmed by our evidence.

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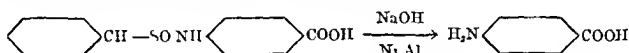
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# A STUDY OF SOME FACTORS INVOLVED IN THE COLORIMETRIC DETERMINATION OF CARONAMIDE

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CARONAMIDE (4' carboxy phenylmethanesulfonamide) inhibits the renal excretion of penicillin and thereby enhances and prolongs the levels of that antibiotic in body fluids<sup>1</sup>. A simple and accurate method for assay of Caronamide is desirable both to facilitate pharmacologic studies and to control dosage. Ziegler and Sprague<sup>2</sup> described a colorimetric method which involves the use of Raney catalyst alloy (powdered Ni-Al) to cleave Caronamide and produce para-aminobenzoic acid (PABA).



The PABA is then determined by Eckert's modification<sup>3</sup> of Bratton and Marshall's method for sulfanilamide<sup>4</sup>. Ziegler and Sprague presented alternate methods applicable to blood serum both of them depending on the reaction cited. One of these (then method II) involves preliminary deproteinizing while the other (method III) which is run upon serum directly is stated to be less accurate but quicker and hence useful where a high degree of accuracy is not needed. In applying these methods to known amounts of Caronamide certain details were found to affect significantly the amounts recovered. The findings are presented in this paper.

## MATERIALS AND METHODS

**Standard Solutions**—'Primary standards' of PABA and Caronamide were prepared by dissolving 0.001161 mole in 20 ml of 5 per cent NaOH and diluting the solutions up to 1 liter with water. These concentrations were chosen because they are each chemically equivalent to the primary sulfanilamide standard solution used in the Bratton and Marshall method. The primary standards were then diluted 1:10 and 1:20 with 5 per cent NaOH in order to bring the intensity of the color formed in the reaction within the optimal range for the spectrophotometric readings. Such dilutions constituted the secondary or working standards, of which 5.0 ml aliquots contained  $580 \times 10^{-6}$  or  $290 \times 10^{-6}$  meq. These amounts if present in 5.0 ml of filtrate obtained by either method II or III of Ziegler and Sprague would correspond to the presence of 15.9 and 7.9 mg per cent of PABA or 33.8 and 16.9 mg per cent of Caronamide in the serum.

Identical spectrophotometric readings may be expected from equivalent amounts of PABA or Caronamide provided the liberation of PABA from

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Carionamide is quantitative and no other substances are formed which influence light absorption materially at 540  $m\mu$ , the wave length at which the PABA was determined. The identity with the sulfanilamide standard substantiated the basic path of the reaction and the derived calculations.

*Other Reagents*—These included pooled human serum or citrated plasma, Raney catalyst, n-octyl alcohol, 6N HCl, 90 per cent ethanol, 0.2 per cent sodium nitrite, 2.0 per cent ammonium sulfamate, and 0.1 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride, the last three in aqueous solutions, freshly prepared.

*Apparatus*—Standardized pipettes and 50 and 100 ml volumetric flasks were used. The 0.5 ml samples were measured with Ostwald-Folin pipettes using a set time for drainage. Light absorption was determined with the Coleman Junior Spectrophotometer with the readings made at 540 millimicrons. The maximum absorption actually is close to 547  $m\mu$ , but the difference is of no significance due to the flatness of the absorption curve in this region.

*Procedures*—The recovery of PABA or Carionamide from mixtures containing known amounts was studied under varied conditions, each step in the determinations being carried out in duplicate. A battery of 50 ml volumetric flasks was assembled and into each were pipetted 5.0 ml of one of the secondary standards. To each flask were then added 2 drops of n-octyl alcohol to minimize foaming during the reaction, and, to one-half of the flasks, 0.5 ml of pooled normal human serum was added in order to observe the effects of serum on the determinations. Raney catalyst was then added in varied amounts, namely "0" (for PABA), 0.125, 0.25, 0.5, and 1.0 gram. The catalyst was added through a funnel slowly in order to avoid excessive heating and frothing\*. The flasks then stood at room temperature (22 to 27° C), except for occasional swirling, for two hours, twenty-four hours, or forty-eight hours, or they were heated in a bath of boiling water for one half hour.

After the mixtures were allowed to react for the desired periods they were diluted with water to 50 ml, shaken, and filtered through No. 42 Whatman paper. Subsequent treatment was essentially as described by Ziegler and Sprague: a 5.0 ml aliquot of filtrate was added to each of two matched cuvettes containing 0.5 ml of 6N HCl, then 0.5 ml of 0.2 per cent  $\text{NaNO}_2$  solution was added to one tube of the pair and 0.5 ml of water added to the other, the latter serving as a blank. Diazotization was allowed to proceed for five minutes before adding 0.5 ml of 2 per cent ammonium sulfamate to both tubes and, after three minutes, 0.5 ml of 0.1 per cent N-(1-Naphthyl)-ethylenediamine dihydrochloride was added to each tube. The color was then allowed to develop in a dark place for thirty minutes, after which the spectrophotometric readings were made.

\*Heat is evolved when Raney catalyst is added to the reaction mixture which will be only slightly warmed however if the catalyst is added slowly allowing several moments for the dissipation of heat between increments. Room temperature reactions as used here imply such conditions.

Additional studies were also made to determine the effects of citrate,  $Al^{+++}$ , and  $Ni^{++}$  on the colorimetric determination and also whether PABA might be absorbed on the catalyst

*Calculations*—The amount of color produced was practically identical if equivalent amounts of PABA were run through the procedure with serum but without catalyst—either hot or cold or for a long or short time. Variations from the mean were less than 0.2 per cent, accordingly the average of these determinations was chosen to represent 100 per cent completeness of the reaction. Equivalent amounts of PABA or Caronamide often gave less intense color in the presence of catalyst. In these instances the recovery was calculated from the ratio of the optical densities. Deviations from the mean of duplicate determinations were usually less than 1 per cent for either Caronamide or PABA provided the amount of catalyst was limited to 0.125 or 0.25 gram.

### RESULTS

It was found that the completeness of recovery of either Caronamide or PABA was affected by the duration of reaction, temperature, presence of serum and amount of catalyst. The findings are summarized in Table I and part of them is shown graphically in Fig. 1. The analytical yield from the mixtures studied was superior when the amount of catalyst was limited to 0.125 or 0.25 Gm. this amount was sufficient for the complete cleavage of Caronamide. Serum enhanced the degree of recovery. In one instance in

TABLE I. RECOVERY OF p AMINO BENZOIC ACID AND CARONAMIDE

REACTION MIXTURE* 50 ML OF 5% NaOH AND			PABA RECOVERED FROM REACTION MIXTURE (%)				CARONAMIDE RECOVERED FROM REACTION MIXTURE (%)			
POOLED HUMAN SERUM (ML)	RANEY CATALYST ADDED (GM)	PABA OR CARONAMIDE (MEQ $\times 10^{-3}$ )	HOURS AT ROOM TEMPERATURE			$\frac{1}{2}$ HR IN BOILING WATER	HOURS AT ROOM TEMPERATURE			$\frac{1}{2}$ HR IN BOILING WATER
			2	24	48		2	24	48	
0.5	0	580	100	--	100	100	0	0	0	0
0.5	0.125	580	99	--	97	96	96	--	94	98
0.5	0.25	580	90	--	86	92	97	--	92	01
0.5	0.5	580	88	76	68	75	91	--	71	76
0.5	1.0	580	85	--	53	53	92	--	49	72
0	0	580	98	--	100	97	0	0	0	0
0	0.125	580	96	--	83	90	93	--	86	92
0	0.25	580	88	--	56	71	87	--	64	73
0	0.5	580	72	46	37	49	74	--	48	53
0	1.0	580	64	--	25	33	70	--	35	42
0.5	0	290	100	--	--	106	0	0	0	0
0.5	0.125	290	100	--	94	101	101	--	84	97
0.5	0.25	290	94	--	77	91	94	--	72	85
0.5	0.5	290	88	54	53	85	79	58	55	88
0†	0.5	290	--	--	--	--	66†	--	--	--
0.5	1.0	290	69	--	25	47	69	--	22	82
0	0	290	97	--	--	96	0	0	0	0
0	0.125	290	89	--	66	86	91	--	72	86
0	0.25	290	72	--	42	59	88	--	40	64
0	0.5	290	55	--	25	48	52	30	27	53
0	1.0	290	46	--	11	37	48	0	11	39

\*Two drops of n octyl alcohol added prior to addition of Raney catalyst.  
†Starch present instead of serum

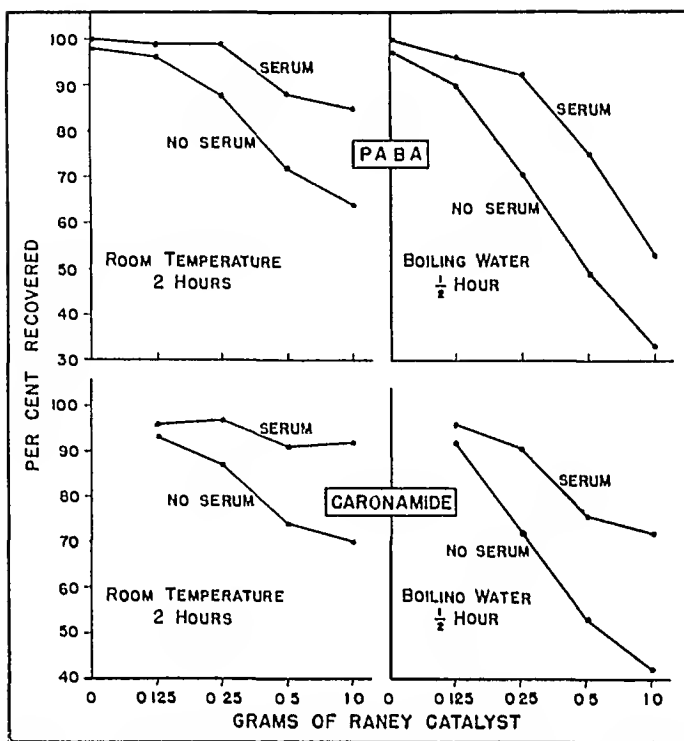


Fig. 1—Recovery of *p*-aminobenzoic acid (PABA) or Caronamide under various conditions (All determinations were made with  $550 \times 10^{-6}$  mEq in the reaction mixture)

which starch was substituted for serum, a "protective" effect was similarly observed. Optimum recoveries were obtained at room temperature and with catalytic contact for only two hours.

Certain factors were found to be of little or no importance: (1) the serum itself was not altered in the procedure so as to produce compounds absorbing light in the region  $540 \text{ m}\mu$ , (2) the presence of citrate had no effect, (3) the pH and total acid of the filtrates prior to diazotization were constant regardless of the amount of catalyst, (4)  $\text{Al}^{+++}$  in amounts greater than those which might accrue from the catalyst did not interfere with the colorimetric reaction and  $\text{Ni}^{++}$  also had no effect, (5) caking of the catalyst when used in larger amounts was present variably but could not be correlated specifically with the results, and (6) the volume usurped by the catalyst was small and would in any case cause an error tending to give falsely high recoveries in experiments involving large amounts of catalyst, which is contrary to the observations.

#### OBSERVATIONS ON SERUM OF A PATIENT RECEIVING CARONAMIDE

Method III and method II of Ziegler and Sprague<sup>1</sup> and certain modifications were applied to the serum of a patient who was receiving large doses of Caronamide. Aliquots of a 1:10 dilution of the patient's serum in 5 per cent NaOH were used for a series of analyses and the amount of catalyst was varied



from 0.125 to 1.0 gram. Reactions were carried out at room temperature for two hours, or in a bath of boiling water for one half hour. The resulting reaction mixtures correspond to those in Ziegler and Spiguel's method III, extended with respect to conditions of time, temperature, and amount of catalyst. Another factor, which at first sight might not be expected to affect the determination was also varied: this was the total volume of the 1:10 serum NaOH solution in contact with the catalyst. Studies were made using 5 ml of 5 per cent NaOH (which contained 0.5 ml serum) in a reaction flask of 50 ml volume, as had been done in the preceding section of this paper, and they were also carried out with 10 ml of 5 per cent NaOH (which contained 1.0 ml serum) in a 100 ml flask essentially the volumes recommended by Ziegler and Spiguel.

In addition the serum was deproteinized with 90 per cent alcohol and filtered as in Ziegler and Spiguel's method II. A 5.0 ml aliquot of the alcohol filtrate, which represented a 1:10 dilution of the serum, was added to 5.0 ml of 5 per cent NaOH in a 50 ml flask and carried through the procedure using various amounts of catalyst either at room temperature or in a bath of boiling water.

**Results**—The Caronamide concentrations obtained with this serum are shown in Table II. It would appear that the results obtained with method II and its modifications compared favorably with those obtained by Method III when the smaller volumes were used. It should be borne in mind, however, that the former values may be as much as 10 per cent in excess of the true values because of the fact that during the filtration some alcohol is lost by evaporation and the solutions become correspondingly more concentrated. A more complete comparison of these methods should include the effect of serum added to the reaction mixture but such a comparison is not possible because of the presence of the alcohol following deproteinization.

Differences were observed in the analytical results when solutions were compared which had similar concentrations but differed in the ratio of volume of solution to the amount of catalyst. Presumably the reaction occurs at the surface of the catalyst and its completion may be determined by a number of

TABLE II RESULTS OF CARONAMIDE DETERMINATIONS DONE BY THE METHODS OF ZIEGLER AND SPIGUEL AND MODIFICATIONS OF SERUM OBTAINED FROM A PATIENT RECEIVING THE DRUG

AMOUNT OF CATALYST (GM)	METHOD III AND MODIFICATIONS USING 0.5 ML SERUM AND 5.0 ML 5% NaOH IN 50 ML FLASK		METHOD III AND MODIFICATIONS USING 1.0 ML SERUM AND 10.0 ML 5% NaOH IN 100 ML FLASK		METHOD II AND MODIFICATIONS USING 0.5 ML SERUM DEPROTEINIZED WITH 5.0 ML 90% ALCOHOL AND ADDING 5.0 ML NaOH IN 50 ML FLASK†	
	ROOM TEMPERATURE (2 HR)	BOILING WATER (½ HR)	ROOM TEMPERATURE (2 HR)	BOILING WATER (½ HR)	ROOM TEMPERATURE (2 HR)	BOILING WATER (½ HR)
0.125	34	33	31	26	28	33
0.25	35	33	31	33	31	33
0.5	34	28	33	29	33	32
1.0	32	24	33	28	32	30

All values are in milligrams per 100 milliliters.

†Evaporation of alcohol during filtration and the resulting increase in the concentration of the solutions may account for as much as 10 per cent of these values.

factors involving the surface area in addition to the concentrations in the overlying solution. Boiling serves to stir the mixtures, and it is possible that mixtures of larger volume at room temperature do not adequately mix when only 0.125 Gm. catalyst is added and gas evolution is curtailed.

In these experiments, which employed serum from a patient receiving Caronamide, the results were not so clear-cut as those obtained with the use of standard solutions of the chemicals. This may be due to the presence of metabolic products of Caronamide. The method of Brodie, Levy, and Bernstein,<sup>2</sup> which employs spectrophotometric measurements in the ultraviolet, was not used here. This method is said to determine Caronamide but not its metabolic products and its use may clarify some of the discrepancies.

#### COMMENTS

These studies confirm the usefulness of Raney catalyst alloy as a basis for the determination of Caronamide. It is desired only to emphasize some of the variables which may influence the reaction and the accuracy of the results.

Eckert<sup>3</sup> has observed that PABA is not fully recoverable from mixtures containing a reducing agent. He noted that the loss of PABA could be nearly 15 per cent in one hour when titanous chloride was present as a reducing agent, and that such mixtures, when heated in a bath of boiling water and then allowed to stand a few hours, sometimes yielded very low recoveries. Eckert also considered coprecipitation of PABA with protein to be a possible explanation but concluded that it was probably not significant since variation of the protein concentration over wide ranges had little effect.

It was shown here that, in the presence of Raney catalyst, PABA was recoverable to varying degrees depending on the experimental circumstances. Whether this loss is due to the partial destruction of PABA, or to adsorption on the surface of the catalyst, is not certain. An attempt was made to leach the "lost" PABA from the catalyst, and this was unsuccessful.

The failure to recover Caronamide in full may be due either to its relationship to PABA or to factors peculiar to Caronamide itself. Some of the possibilities which suggest themselves are the formation of compounds other than PABA upon reduction, or adsorption of Caronamide itself, or competing rates of reduction, destruction and adsorption of the reagents. These details need clarification.

It was shown here that the analytical recovery of PABA or Caronamide from known mixtures of these chemicals can be made better than 90 per cent. It is necessary only to limit the amount of catalyst employed, and to carry out the reaction at room temperature for a short time. The presence of serum enhances the recovery.

The mixtures employed corresponded in the total amounts and concentrations of PABA or Caronamide to those customarily produced during a routine analysis of serum samples from patients according to Ziegler and Sprague's methods. When the method which was found to work best on the synthetic

mixture was applied to the analysis of a serum sample from a patient receiving Caronamide, the comparative results were somewhat less convincing. It appears, however, that the greatest yield was obtained when the reaction was carried out at room temperature and with the smaller amounts of catalyst in the smaller volume.

On the basis of the present findings the following modification of Ziegler and Sprague's method for serum Caronamide determination is suggested.

An accurate 0.5 ml sample of serum is diluted with 5 ml of 5 per cent NaOH (or a 5 ml aliquot of 1:10 serum 5 per cent NaOH solution is used). 2 drops of n-octyl alcohol are added and then 0.125 or 0.25 Gm of the Raney catalyst. After standing two hours at room temperature the mixture is diluted to 50 ml with water, filtered, and conjugated in the usual fashion.

Even small variations from the method as stated affect the determinations. Comparisons of one method with another or with its modifications, should be evaluated with this in mind.

#### SUMMARY

1 The analytical method of Ziegler and Sprague for the colorimetric determination of Caronamide together with modifications has been used for analysis of known solutions of Caronamide and para aminobenzoic acid and for serum from a patient receiving Caronamide.

2 The duration of the reaction, the temperature at which it is carried out, the amount of catalyst and the presence of serum all were found to influence markedly the degree of recovery of Caronamide or para aminobenzoic acid.

3 Recoveries were better in general when the reaction was carried out with serum present at room temperature for two hours and with a smaller amount of catalyst than has hitherto been recommended.

4 A modification of Ziegler and Sprague's method for serum Caronamide determination is suggested on the basis of these findings.

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## DIMETHYLETHER OF *d*-TUBOCURARINE IODIDE

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**D**IMETHYLETHER of *d*-tubocurarine iodide is the reaction product of methyl iodide with *d*-tubocurarine in methanol potash. It involves the methylation of the two phenolic hydroxyl groups and an exchange of iodine for chlorine ions. The compound was first prepared by King<sup>1</sup>. Several investigators<sup>2-6</sup> reported that the methylated compound was considerably more active than the natural alkaloid, *d*-tubocurarine. Stoelting, Giat, and Viena<sup>7</sup> found that in man dimethylether of *d*-tubocurarine iodide was highly effective in relaxing voluntary muscles during light anesthesia, and that the required dose was much smaller than that of *d*-tubocurarine. It thus becomes desirable to extend the pharmacologic studies with the methylated alkaloid in order to survey its full action and insure its safety for human use.

The sample of dimethylether of *d*-tubocurarine iodide employed in the present work was anhydrous and in the form of trihydrate, melted at 267 to 270° C. with decomposition, and had a specific rotation of  $[\alpha]_D^{25} + 150^\circ$  in aqueous solution. The substance was easily soluble in water. A stock solution of 1:1,000 was prepared, and suitable dilutions were made from it. Wherever possible, the drug was compared with *d*-tubocurarine chloride in the form of pentahydrate.

**1 Neuromuscular Action.**—Dimethylether of *d*-tubocurarine was tested for its curarizing effect in the cat and rat. Certain suggestions of procedure were made by Professor G. H. Acheson, University of Cincinnati, based on Van Maanen's publication<sup>8</sup>. Briefly, after the animal was anesthetized with Seconal Sodium (Lilly) (sodium propylmethylethylallyl barbiturate acid), the gastrocnemius muscle was exposed, and the tendo calcaneus (Achilles tendon) severed from its insertion. The sciatic nerve was isolated in the thigh and crushed at the site of emergence from the vertebral column. All its branches were cut except those supplying the gastrocnemius muscle. The skin was sewed together to prevent drying.

For recording of contractions, a hook was inserted in the tendo calcaneus and fastened to an isotonic muscle lever. A shielded platinum electrode was placed on the sciatic nerve just distal to the point of crushing. In the rat, a single stimulus of 50 volts from a stimulator (Electrodyne) caused a maximal contraction. This could be repeated every two seconds in each animal for long periods of time. In the cat, it required shocks of 50 volts at a rate of fifty per second for two seconds to produce a maximal, smooth, sustained contraction. This could be repeated every thirty seconds in each cat for intervals of several hours.

After a control period of repeated stimulation, resulting in a series of uniform contractions, the methylated alkaloid was injected intravenously. Observations were continued until recovery occurred. In several animals, the injections were repeated from one to three times. The paralyzing action of the dimethylether is exactly the same as that of *d* tubocurarine, differing only in degree. In six rats, the new alkaloid completely inhibited the contractions in the dose of 24  $\mu$ g per kilogram reduced the contractions by 76 to 91 per cent in the dose of 16 to 20  $\mu$ g per kilogram, and by 28 to 72 per cent in the dose of 14  $\mu$ g per kilogram. The duration of action of all doses was approximately the same, averaging thirty three minutes. With *d* tubocurarine chloride in six other rats, complete inhibition of contractions occurred with a dose of 112 to 128  $\mu$ g per kilogram and reductions to the extent of 24 to 28 per cent with a dose of 64 to 96  $\mu$ g per kilogram. The average duration of action of these doses was thirty five minutes. No effect was noted in smaller doses. Thus as previously reported<sup>9</sup> the methylated alkaloid is much more potent than the natural alkaloid.

Six cats receiving the dimethylether in doses ranging from 20 to 24  $\mu$ g per kilogram showed a maximal reduction of 81 per cent in the amplitude of contractions. One of them died from the larger dose in eleven minutes. The average duration of action in the remaining five cats was sixty two minutes. A dose of 10  $\mu$ g per kilogram was ineffective. It was not possible to produce complete inhibition of contractions without causing death. In seven other cats *d* tubocurarine chloride in the dose of 100 to 174  $\mu$ g per kilogram caused on the average, a maximal reduction of contractions by 70 per cent. Two of the cats died of respiratory failure. The average duration of action in the surviving animals was forty three minutes. Fig. 1 contrasts the effects of the two substances. It appears that in the cat the methylated alkaloid is not only more potent than *d* tubocurarine, but also longer acting.

Garcia de Jalon<sup>9</sup> reported that *d* tubocurarine chloride, quantitatively, antagonized the acetylcholine contracture of the frog's rectus abdominis. A similar study was made with dimethylether of *d* tubocurarine in the present work. The rectus muscle of *Rana pipiens* was immersed in a volume of 10 c.c. of aerated Ringer's solution, and its contractions were registered on a kymographine paper. Acetylcholine amounting to 5  $\mu$ g was added to the bath every ten minutes and washed out twice each time at the end of the fifth minute. This resulted in a series of uniform maximal contractions. When various volumes of a 0.0001 molar solution of the methylated alkaloid were placed in the bath one and one half minutes before the scheduled doses of acetylcholine, the amplitude of contractions became reduced. The reduction of acetylcholine contractions was proportional to the amount of the drug superimposed. A comparison was made with *d* tubocurarine also in 0.0001 molar solution on the same muscle preparations by alternating with the dimethylether. In a total of twenty eight parallel observations, dimethylether of *d* tubocurarine was proved to be equally as active as *d* tubocurarine. Apparently, mole for mole *d* tubocurarine and its dimethylether have the same power to inhibit the acetylcholine contraction of the frog's rectus muscle.

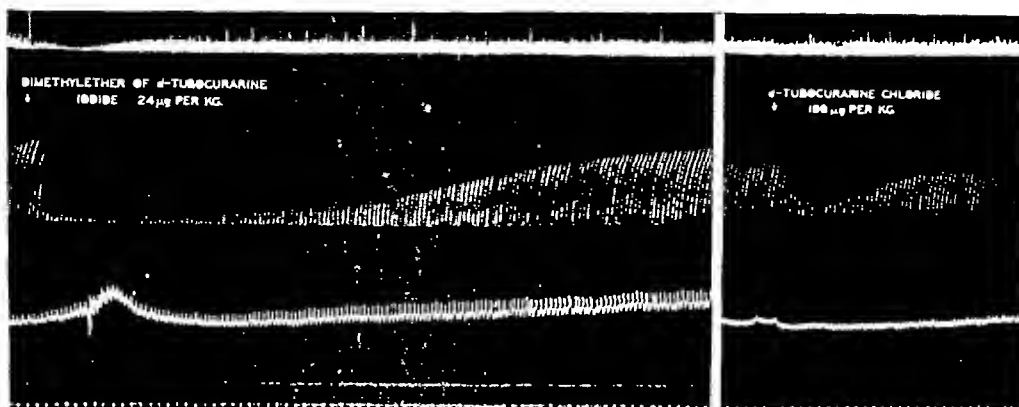


Fig 1—Action of dimethylether of *d*-tubocurarine iodide and *d*-tubocurarine chloride on the gastrocnemius sciatic nerve preparation

The tracings were made from a cat weighing 3.647 kilograms anesthetized with Seconal Sodium (35 mg per kilogram). The curves from top to bottom are respiratory movements, gastrocnemius contractions, carotid blood pressure, baseline and time in minutes. The sciatic nerve supplying the gastrocnemius muscle was stimulated every thirty seconds (see details in the text). Both alkaloids were injected intravenously. The time for full recovery of gastrocnemius contractions following dimethylether of *d*-tubocurarine iodide was 78 minutes and that following *d*-tubocurarine chloride 30 minutes.

**2 Assay in Rabbits**—Like *d*-tubocurarine, the dimethyl-ether produces a head drop of the rabbit by paralysis of the neck muscles. The response of this animal is so uniform that it can be used advantageously for assaying purposes, as already advocated by Bennett and co-workers<sup>10</sup>. By running a cross over test in a group of thirty rabbits, dimethylether of *d*-tubocurarine iodide, injected intravenously, was found to be almost eight times as active as *d*-tubocurarine chloride, as measured by the median effective dose ( $ED_{50}$ ). The rest period between the days of injection was five days. The combined data are shown in Table I. Two separate, new lots of the dimethylether were assayed and found to have  $ED_{50}$ 's of  $15.9 \pm 1.4$  and  $16.6 \pm 1.2$   $\mu$ g per kilogram, indicating the uniformity of the product.

Since a dose of 20  $\mu$ g of dimethylether of *d*-tubocurarine iodide produced the same response as one of 150  $\mu$ g of *d*-tubocurarine chloride, per kilogram (see Table I), a special cross-over test was carried out with the same doses in another group of twenty rabbits, for the comparison of onset and duration of

TABLE I COMPARISON OF POTENCY BY INTRAVENOUS INJECTION BETWEEN *d* TUBOCURARINE AND ITS DIMETHYLETHER BY THE RABBIT CROSS OVER TEST

ALKALOID	DOSE ( $\mu$ G PER KG)	NO. IN HEAD DROP	MEDIAN EFFECTIVE DOSE $\pm$ STANDARD ERROR ( $\mu$ G PER KG)
		NO. USED	
Dimethylether of <i>d</i> tubo curarine iodide	15.0	4/10	$15.8 \pm 0.8$
	17.5	7/10	
	20.0	9/10	
<i>d</i> Tubocurarine chloride	100.0	2/10	$123.0 \pm 7.0$
	125.0	4/10	
	150.0	9/10	

action. A stop watch was employed. With the methylated alkaloid, 20  $\mu$ g per kilogram by vein, the average time required for the appearance of head drop was 1.02 minutes, and that for restoration of the head position, 18.25 minutes. With *d* tubocurarine, 150  $\mu$ g per kilogram intravenously it took an average of 1.75 minutes for the head to drop, and an average of 13 minutes for the animal to raise the head again. It appears evident that in the rabbit the methylated alkaloid has a shorter onset, but a longer duration, of action than *d* tubocurarine.

**3 Decurarization**—Burke and associates<sup>11</sup> recently reported the antidotal action of neostigmine against *d* tubocurarine. In our work, neostigmine methylsulfate was found to have a limited value. For example, the median lethal dose ( $LD_{50}$ ) of dimethylether of *d* tubocurarine iodide in twenty-five rabbits by vein was  $31.5 \pm 1$   $\mu$ g per kilogram. When neostigmine methylsulfate in the dose of 50  $\mu$ g per kilogram was simultaneously injected also by vein the  $LD_{50}$  of the methylated alkaloid in forty new rabbits was raised to  $53.5 \pm 1.9$   $\mu$ g per kilogram. However, when the same dose of neostigmine was given three minutes after the injection of dimethylether of *d* tubocurarine iodide in a third group of fifteen rabbits the  $LD_{50}$  of the latter became  $38.1 \pm 4.6$   $\mu$ g per kilogram. In other words, the antidotal action of neostigmine rapidly diminishes after the methylated alkaloid has been administered.

Further evidence was obtained regarding the narrow efficacy of neostigmine in six etherized dogs. The depression of respiratory amplitude and rate following small doses of dimethylether of *d* tubocurarine iodide, 5 to 10  $\mu$ g per kilogram, was easily removed by intravenous injection of neostigmine methylsulfate in the dose of 50 to 100  $\mu$ g per kilogram. The latter however was only capable of delaying death in the etherized dog if the dose of the curari form alkaloid was increased to 20  $\mu$ g per kilogram or more.

The most effective measure to revive dogs from large and fatal doses of dimethylether of *d* tubocurarine iodide was artificial respiration. It restored their respiration after neostigmine had failed. The work was repeated with equivalent doses of *d* tubocurarine and similar results were obtained.

**4 Synergism With Anesthetics**—Of seven dogs anesthetized with ether three died following intravenous injection of dimethylether of *d* tubocurarine iodide in the dose of 20 to 25  $\mu$ g per kilogram and four survived a dose of 4 to 8  $\mu$ g per kilogram. Of five dogs anesthetized with pentobarbital sodium, three died with doses of 60 to 65  $\mu$ g per kilogram while the remaining two lived with smaller doses. No artificial respiration was applied to any of these animals. When the drug was injected intravenously into four nonanesthetized dogs two on a dose of 75  $\mu$ g per kilogram lived without artificial respiration and one of the other two on a dose of 100  $\mu$ g per kilogram lived with artificial respiration. It was also observed in two cats and two rats that a supplementary dose of a barbiturate equal to one tenth of the anesthetic dose given during curarization by the dimethylether was fatal to all of them. These results indicate very clearly that simultaneous administration of the fixed anesthetics and dimethylether of *d* tubocurarine kills animals in smaller doses than when each is injected alone.

In a similar series of eight dogs, four anesthetized with ether and four with pentobarbital sodium, *d*-tubocurarine was lethal in smaller amounts to them than to nonanesthetized animals. Such a synergism is conceivable because when the respiratory center is depressed by the general anesthetics, the muscles of respiration tend to fail more readily with curarizing drugs such as *d*-tubocurarine and its dimethylether.

*5 Action on Respiration, Blood Pressure, Uterus, and Intestines*—In both the anesthetized cat and dog, sublethal doses of dimethylether of *d*-tubocurarine caused a transient decrease of respiration, both in amplitude and rate—the larger the dose, the more the decrease. The decrease in amplitude is clearly illustrated in Fig. 1.

The response of blood pressure was not uniform, as shown in six cats and fourteen dogs. In general, doses of 5 to 10  $\mu\text{g}$  per kilogram were without effect. Occasionally, doses of 20 to 24  $\mu\text{g}$  per kilogram were followed by a rise of blood pressure, particularly in those animals in which the initial pressure was low. A terminal fall of pressure ending in death occurred with lethal doses—some times preceded by a slight rise.

The uterine activity of eight, post partum, anesthetized rabbits was recorded through a pulley. It was observed that dimethylether of *d*-tubocurarine iodide in the dose of 10 to 20  $\mu\text{g}$  per kilogram produced a slight to moderate increase of uterine contractions in four animals, no effect in three, and questionable relaxation in one.

Three observations were made on the skin-covered intestinal loops of two trained dogs, the operative technique of which was described by Wakim and Mason<sup>12</sup>. Intravenous injection of the methylated alkaloid, 10 to 15  $\mu\text{g}$  per kilogram, caused no changes in peristaltic movements.

*6 Toxicity*—The median lethal doses of dimethylether of *d*-tubocurarine were determined by intravenous injection in the mouse, rat, guinea pig, and rabbit—comparisons being made at the same time with *d*-tubocurarine. Death was unquestionably due to respiratory failure, preceded by asphyxial clonic convulsions. As shown in Table II, the methylated alkaloid is less toxic to the mouse, but more toxic to the rat, guinea pig, and rabbit, than *d*-tubocurarine.

TABLE II COMPARISON OF TOXICITY BY INTRAVENOUS INJECTION BETWEEN *D* TUBOCURARINE AND ITS DIMETHYLETHER

ALKALOID	ANIMAL	NUMBER USED	DOSE RANGE ( $\mu\text{G}$ PER KG)	LD <sub>50</sub> $\pm$ STANDARD ERROR ( $\mu\text{G}$ PER KG)
Dimethylether of <i>d</i> tubo curarine iodide	Mouse	60	180.0-330.0	238.0 $\pm$ 13.0
	Rat	28	27.5-50.0	34.5 $\pm$ 1.7
	Guinea pig	22	50.0-62.0	50.4 $\pm$ 2.3
	Rabbit	25	27.5-36.5	31.5 $\pm$ 1.0
<i>d</i> Tubocurarine chloride	Mouse	50	125.0-200.0	153.0 $\pm$ 5.0
	Rat	24	80.0-125.0	109.0 $\pm$ 5.0
	Guinea pig	15	62.0-80.0	65.7 $\pm$ 3.5
	Rabbit	15	200.0-250.0	221.0 $\pm$ 10.0

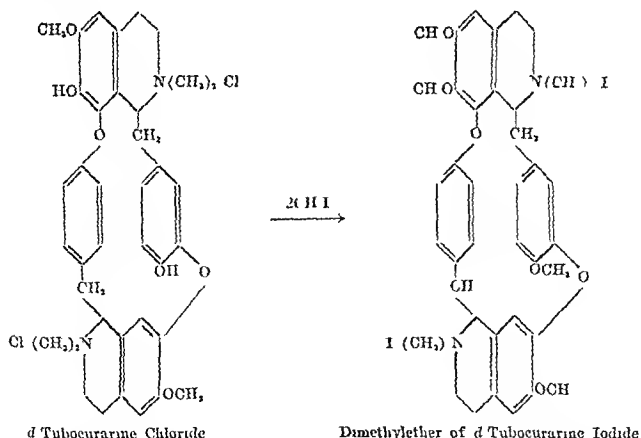


A group of eight rabbits were each injected intravenously with an ED<sub>50</sub> for head drop per day for two weeks except Saturday and Sunday. The neck muscles of all animals were paralyzed with complete recovery, showing no development of tolerance. The day following the tenth dose, each rabbit was given a dose of 365  $\mu$ g per kilogram. Six out of eight succumbed—further evidence of no tolerance. At necropsy visceral organs including the heart, lungs, liver, spleen, stomach, intestines, kidneys, thymus and adrenal glands, were found normal both grossly and microscopically.

## DISCUSSION

From the foregoing data which agree with those of Collier, Paris and Woolf<sup>6</sup> it is obvious that dimethylether of *d* tubocurarine iodide, like its parent alkaloid, has a blocking effect on the motor end plate of striated muscles of the rat and cat. It reduces the contraction of the frog's abdominal muscle induced by acetylcholine. According to Acheson<sup>13</sup> such a blocking agent "prevents depolarization of acetylcholine by competitive inhibition." Depolarization by neostigmine limited in extent is probably due to the protection from hydrolysis of the acetylcholine produced by the motor nerve.

The remarkable feature of this product is that methylation of the two phenolic groups of *d* tubocurarine as shown below results in an increase of potency close to eightfold as measured by the rabbit head drop method—confirming approximately the results of Wintersteiner and Dutcher,<sup>2</sup> and Marsh and co-workers.<sup>4, 5</sup>



This is not due to the change of the molecular size because the ratio between dimethylether of *d* tubocurarine iodide and *d* tubocurarine chloride is 12 to 1. In the rabbit and cat, the duration of action of the methylated alkaloid is sub

stantially longer than that of *d*-tubocurarine. The English workers<sup>6</sup> also found the former longer acting in the rabbit than the natural alkaloid. They further observed that, in the rat, the dimethylether was completely excreted in urine, while *d*-tubocurarine was partially eliminated by the renal route.

From a therapeutic point of view, dimethylether of *d*-tubocurarine iodide can adequately replace *d*-tubocurarine chloride in anesthesia, as already reported by Stoelting, Graf, and Vienna.<sup>7</sup> Its longer action in equivalent doses is an advantage. Because of the synergism between anesthetics and curarizing agents, special caution must be exercised in the simultaneous administration of the two classes of drugs. In the treatment of an overdose of dimethylether of *d*-tubocurarine iodide, artificial respiration is far more reliable than neostigmine. This is also true with *d*-tubocurarine.

#### SUMMARY

1 Dimethylether of *d*-tubocurarine iodide inhibits electrically induced contractions of the gastrocnemius muscle of the rat and cat. It reduces the effect of acetylcholine on the frog's rectus muscle.

2 Compared with *d*-tubocurarine chloride, it is close to eight times as potent, the rabbit head-drop method being used. The duration of action is longer with the methylated product in the rabbit and cat than with *d*-tubocurarine.

3 The intravenous toxicity of dimethylether of *d*-tubocurarine iodide by a single injection in the mouse, rat, guinea pig, and rabbit, has been determined and compared with *d*-tubocurarine chloride. Repeated injections do not cause visceral damage.

4 Like *d*-tubocurarine, the dimethylether is synergistic with ether and barbiturates. Artificial respiration is a superior antidote to neostigmine methylsulfate in the treatment of overdosage. This is also true with *d*-tubocurarine.

5 The response of arterial blood pressure in the anesthetized cat or dog to sublethal doses of the dimethylether, injected intravenously, is not uniform—sometimes no change and other times a drop or a rise. A terminal fall of blood pressure occurs after lethal doses, secondary to respiratory failure. In the post-partum rabbit, the methylated alkaloid frequently contracts the uterus in situ. It has no action on the intestines of the trained dog.

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# HEREDITARY ANGIONEUROTIC EDEMA, WITH A CASE REPORT

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ANGIONEUROTIC edema is a condition which is alarming to the patient and baffling to the physician. First described by Milton<sup>1</sup> in 1876 as "giant urticaria," the malady usually is called Quincke's edema after Heinrich Quincke,<sup>2</sup> the physician from Kiel. His paper in 1882 on "acute circumscribed edema" has been accepted by posterity.

The majority of patients are consistent in their stubborn failure to respond to the usual methods of treatment, and probably not over 50 per cent of cases are solved in regard to etiology. Cooke<sup>3</sup> states that when the mechanism is discovered, it frequently appears to be related to a current infection in some part of the body, while Cohen<sup>4</sup> suggests that many cases may be traced to drug sensitivity. There remains, however, that large group in whom the cause is a mystery. While the hereditary element in some cases of angioneurotic edema was recognized from the start, a family relationship is not the usual finding.<sup>3</sup> Most attacks occur in individuals who have no family history of edema or accompanying personal allergic manifestations. Lennon<sup>5</sup> nevertheless called attention to ten patients, all women, in whom he found frequently associated histories of asthma, hay fever, or migraine. There is some uncertainty concerning its neurotic origin, but the swelling frequently may be traced to neuropathic families.<sup>6</sup>

We wish to consider the familial type cases which, although a smaller group, seem to have particularly severe symptoms and which are prone to develop glottic edema which often leads to death.<sup>7</sup> The outstanding reported cases of hereditary angioneurotic edema are well summarized by Bulloch,<sup>8</sup> who reviewed the twenty-eight families described in the literature by 1909, and more recently by Cockayne<sup>9</sup> and Gates.<sup>6</sup> For convenience, some of the papers of interest will be reviewed here. Quincke's original work,<sup>2</sup> in which he reported the swelling in a man and two of his children, alerted later clinicians to the hereditary possibility. Osler's paper<sup>10</sup> in 1888 outlined a family in which twenty-two cases of angioneurotic edema progressed in a dominant manner through five generations with only one skip. Enso<sup>11</sup> published findings in 1904 from a family in which twenty members were affected in four generations (twelve male members, eight female members). Of these, twelve died from suffocation during an attack. Five years later, Enso had collected forty-nine cases (twenty-eight male patients, twenty-one female patients), in six generations in which the heredity was following a dominant pattern, with one skip. Crowder and Crowder<sup>7</sup> in 1917 described twenty-eight cases (fourteen men, fourteen women) which passed

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through five generations in a simple strict dominant fashion. There were fifteen deaths in this family due to glottic edema. In 1920 Cameron<sup>1</sup> cited eight cases in three generations in which three members died of suffocation. Dunlap and Lemon<sup>12</sup> in 1929 pointed out eleven cases in which the inheritance was strictly dominant through four generations. Oakley<sup>9</sup> in 1933 collected the reports of thirty nine families in which there were 257 cases of angioneurotic edema. Of these, twenty four families were described completely enough for him to estimate that the proportion of affected to normal members was 182 involved to 206 uninvolved. The sex ratio of those with the condition was about 15 men to 1 woman.

#### CASE REPORT

The high incidence of death due to glottic edema is fair evidence of therapeutic failure, and the fact that the exact etiology and mechanism of congenital angioneurotic edema are still unknown justifies further case reports. Our case is taken from a family in which the hereditary form of angioneurotic edema was demonstrated in three and possibly six members of the family, and in which the trait was passed through the generations in a regular dominant pattern. The patient was a 24 year old white man of Italian extraction and was first seen in the allergy clinic of the University Hospital on March 6 1946. He gave a history of transient diffuse swellings which had occurred intermittently since the age of 7. The frequency of the attacks varied from once in three weeks to daily occurrence. Each bout lasted from one to three days. Commonly the face, throat, arms and legs were involved but lesions also occasionally appeared on the trunk and genitalia. The swellings were described as frequently following a blow to the affected area, although trauma was not a necessary antecedent. The edematous areas were usually several inches in diameter diffusely indurated and erythematous. Subjectively there was described a sense of tension in the site involved.

In the past there also had been episodes of gaseous abdominal distention and eructation often associated with nausea and vomiting and usually occurring after meals. He had experienced only the usual childhood diseases. There was no history of allergic rhinitis, asthma or eczema. An appendectomy had been performed in March, 1943, presumably following one of the gastrointestinal episodes. The family history was of interest as outlined in Fig. 1.

When the patient was first seen in the allergy clinic he demonstrated diffuse swelling of the left upper arm and of the right knee. The general physical examination revealed a well developed and well nourished young man. Blood pressure was 120/70, and pulse 60. A sebaceous cyst 0.5 by 1.0 cm. was found behind the left ear. The anterior portion of the nasal septum was markedly deviated to the left and moderate lymphoid hyperplasia was noted in the nasopharynx. The heart, lungs, and abdomen were normal.

In April, 1946, study of the blood showed 13.3 Gm. of hemoglobin per 100 c.c., 4,700,000 red blood cells per cubic millimeter, and 9,800 white blood cells per cubic millimeter with a differential count of 57 per cent neutrophils, 2 per cent eosinophils, 18 per cent small lymphocytes, 15 per cent large lymphocytes, and 8 per cent monocytes. The hematocrit was 41.0, the mean corpuscular volume was 87, and the sedimentation rate was 8 mm. per hour (Wintrobe method). Examination of the urine showed no abnormality. A routine microfluorogram of the chest was read as normal and the blood Kahn serologic reaction for syphilis was negative. A search for foci of infection during this time disclosed no evidence of infection of the mouth, pharynx, gall bladder, nasal accessory sinuses, or genitourinary tract.

The patient was skin tested with 126 allergenic materials. The prick tests were entirely negative in reaction except for a questionable reaction to Endo house dust and caddis fly. On intradermal testing there was read a 4 plus reaction (wheal, pseudopod, and erythematous halo) to Endo house dust, stock house dust, caddis fly, and mugwort. A 3 plus reaction was recorded for orris root, corn smut, quince seed, burweed, marsh elder, wormwood, poplar, willow, pepper, and barley. No correlation could be made between the clinical picture and the skin reactions.

asthma, migraine, eczema, or urticaria. The edema encountered in the first group generally is more severe. It shows a more regular, nonskipping pattern of dominant inheritance and gives least evidence of being associated with a hypersensitive state, except for physical factors, such as heat, cold, and trauma. Gates<sup>6</sup> describes angioneurotic edema as a very irregular dominant condition as viewed by the geneticist. The inheritance, he feels, is not recessive, but rather is incompletely or irregularly dominant.<sup>14</sup> Cockayne<sup>9</sup> suggests that the basic cause of the edema is protein hypersensitivity, particularly in patients with associated asthma and urticaria. Fineman<sup>12</sup> looks upon the condition as nonallergic and proposes that it is caused by physical and emotional exciting agents. In our case we were unable to point to any allergic basis for precipitation of the attacks of edema. The findings in the family we are reporting lead us to believe that we are dealing with an example of a dominantly inherited classical form of Quincke's edema. We previously instructed the two members (the patient and his sister, *II-9* and *II-4*) that the disorder would be expected in half of their respective offspring. Onset of bouts of edema may occur at any age, but usually appear before the age of 20. In this family, ages at first attack were 2, 10, and 7 years, respectively (*II-1*, *II-4*, *II-9*). Edema of the gastrointestinal tract is reported commonly and evidently occurred in the patient (*II-9*), his sister (*II-4*), and his father (*I-1*). As to whether the father never experienced subcutaneous swellings, or whether these occurred in early life and later were replaced by abdominal attacks, we can only speculate, but both such clinical courses have been described in certain affected members of reported families. When encountered without previous history of external swellings, a localized angioneurotic edema of the abdominal viscera has been mistaken frequently for appendicitis, cholecystitis, and other acute illnesses, and it is possible that such may have been the explanation for *II-2*'s operation. However, evidence for involvement of angioneurotic edema in this child and his sister (*II-3*) must be regarded as questionable.

Brain and meningeal symptoms have been described in cases of angioneurotic edema<sup>16</sup> and this complication has been suspected of causing death in one member of Halsted's<sup>17</sup> family, so there is basis for wondering if the cephalopathy described in our patient's father (*I-1*) might not have been caused by cerebral edema. Epistaxis was a frequent finding in Enson's<sup>11</sup> family. But the most striking features of classical Quincke's disease are as we have said, the severity of symptoms and the high proportion of deaths which result from edema of the larynx and glottis. In the family we studied, *II-1* undoubtedly succumbed in this way, and it seems quite likely that *II-3* did likewise. Proved cases of laryngeal angioneurotic edema in infants are recorded,<sup>18, 19</sup> and many suspicious cases are recounted in other published families. Cotterman<sup>20</sup> has pointed out that of the 170 cases of Quincke's disease contained in the twenty-eight families reviewed by Bulloch, fifty-three deaths are recorded, of which thirty-six or 68 per cent were attributed to edema of the larynx. The age at the time of suffocation is specified in twenty-four of these cases and the mean age is 34.4 years, with standard deviation 18.4 years. With such an early average

age of death, it seems quite probable that some selection is operative against the gene for Quincke's disease and that the condition should tend to disappear. But the incidence of the disease seems to be holding steady and therefore the supply of genes for Quincke's disease must be replenished by the process of mutation. Consequently Cotterman advances the thought provoking question which was first suggested by Cockayne<sup>9</sup> which entertains the theory that a considerable proportion of the reported sporadic cases of angioneurotic edema may be the recipients of mutations and thus may constitute the potential starting members of new family lines of this disease.

An interesting additional discovery in our patient's family was another rare hereditary trait namely blood group A,B. The study of this condition is continuing.

#### SUMMARY

Angioneurotic edema or Quincke's edema usually is not hereditary but there is a small group in which the condition is inherited and in which the trait is passed from one generation to the next in a dominant manner. This group experiences remarkably severe symptoms and death is not unusual during an attack. A case is presented from a family in which the edema definitely affected three and possibly three additional members of two generations. Treatment with histamine may have afforded some protection as long as it was administered and Benadryl given intravenously as soon as symptoms began may have lessened the severity of attacks but no measure was adequate in protecting the patient from a fatal attack of edema. At present we can only speculate about the apparently inherited flaw which permits the outpouring of edema fluid with no obvious provocation.

The authors wish to acknowledge the extensive cooperation of Dr C A Alexander of Kalamazoo for the follow up on the patient's history and of Dr Charles W Cotterman of the University of Michigan Heredity Clinic for the genetics investigation of the family involved and for his many helpful suggestions.

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# FACTORS INFLUENCING THE PRODUCTION OF ANAPHYLAXIS IN GUINIA PIGS WITH WEAKLY ANTIGENIC PROTEIN HYDROLYSATES

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**I**NTRODUCTION of the concept of intravenous protein alimentation, during conditions in which the oral route is temporarily impractical or impossible, raised many questions in the field of applied and clinical physiology. One important problem was the question of potential antigenicity of partially hydrolyzed proteins. With the possibility of repeated and intermittent use of such materials in human beings, the elimination of all antigenic characteristics becomes important if not imperative because of the unpredictable dangers of sensitization and anaphylaxis.

Evidence is available in the literature<sup>1, 2</sup> to indicate that relatively small peptide aggregates may possess some degree of antigenicity. In the acid hydrolysis of proteins the occurrence of such molecular size is inevitable at some stage of the breakdown. In the case of fibrin, which itself is antigenic,<sup>3, 4, 5</sup> the intermediate degradation products may be only weakly antigenic. The antigens if present can be specific entities and may not produce antibodies that cross react with the parent material or vice versa. Such was observed in preliminary experiments attempting to test for the presence of antigens by employing an alkaline solution of fibrin either as the sensitizing or shocking agent and using an acid partial hydrolysate of fibrin as the corresponding material for shocking or sensitizing respectively. No anaphylactic symptoms were demonstrable nor were positive results obtained in similar experiments using blood plasma and fibrin hydrolysate reciprocally. These results are similar to those obtained by Landsteiner with digested egg proteins,<sup>6, 7</sup> but differ from experiments with some enzymatic hydrolysates of sheep serum or casein in which the parent material or even skim milk may better be used for shocking animals in instances of anaphylactic sensitivity induced by an antigenic hydrolysate. The failure of fibrin to behave similarly made it necessary to use the hydrolysate itself when the latter was suspected of being antigenic.

Since several techniques currently used in testing for antigenicity of protein hydrolysates uniformly yielded negative or very unsatisfactory results in the production of an anaphylactic state in guinea pigs, further experiments were conducted with a view to testing various procedures when a hydrolysate of known antigenicity was employed. In the experiments to be described, (unless otherwise specified) all were conducted with the same material, an acid partial hydrolysate (5 per cent solution) of beef fibrin containing 30 per cent free amino acid nitrogen and all shocking doses were administered via the dorsal penile vein.

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The following methods were carried out (a) One intraperitoneal injection of the 5 per cent solution containing the equivalent of 500 mg (dry weight) of material was given to each of twelve animals. Three weeks later, the injection of a challenging dose containing 100 mg of the same material failed to produce anaphylactic symptoms in any of the animals (See Table I). The unsatisfactory results attained by means of this simple but common procedure illustrate the difficulties involved in demonstrating antigenicity.

TABLE I EFFICACY OF VARIOUS METHODS FOR SENSITIZATION BY A WEEKLY ANTIGENIC PROTEIN HYDROLYSATE

	NUMBER OF INJECTIONS	AMOUNT* (MG)	INCUBATION PERIOD† (DAYS)	NUMBER OF ANIMALS TESTED	ANAPHYLACTIC REACTIONS	PER CENT SUCCESS
a	Single injection	500	21	12	0	0
b	3, at 2 day intervals	250	21	8	0	0
c	3, at 5 day intervals	250	28	19	2	11
d	6, at 2 to 3 day intervals	200	31	92	59	64
	1 (booster) ‡	200	10	72	50	70
e	1, alum ppt	750§	21	12	0	0
	1, alum ppt	750	28	7	3	43
	1, alum ppt	750	31	14	5	36
	1, alum ppt	1,000	31	8	5	63
	1 (booster)	200	10	8	8	100

\*Dry weight basis

†After last injection

‡Given thirty-one days after last sensitizing dose

§Alum precipitate from 750 mg

TABLE II INFLUENCE OF DEGREE OF HYDROLYSIS ON RELATIVE ANTIGENICITY

LOT NUMBER	FREE AMINO ACID NITROGEN (PER CENT)	TOTAL GUINEA PIGS TESTED	ANAPHYLACTIC REACTIONS			
			NEGATIVE	NONFATAL	FATAL	PER CENT POSITIVE REACTIONS
I	28	13	2	8	3	85
II	35	15	11	3	1	27
III	41	15	10	4	1	33
IV	47	15	12	3	0	20
V	55	11	11	0	0	0

(b) Three intraperitoneal injections, each containing 200 to 250 mg of material, were given at two day intervals to eight animals. No anaphylactic results were obtained when the animals were challenged after three weeks (Table I). An occasional, nonfatal reaction was observed, however, after four or six weeks.

(c) Three intraperitoneal injections, each containing 250 mg of material, were given at five-day intervals to nineteen animals. After four weeks, anaphylactic reactions were demonstrable in only 11 per cent of the animals challenged with 250 to 500 mg of material (Table I).

(d) Ultimately, a procedure was devised which produced satisfactory sensitization in a much higher percentage of the animals. Doses of 4 to 5 cc of the solution (200 to 250 mg of material) were injected intraperitoneally into healthy male guinea pigs (250 to 400 grams) six times during a two-week period. The animals then were not used for at least thirty to thirty-one days

For shocking each animal was challenged with doses of 3 to 12 cc (150 to 600 mg) of the sensitizing material and it was found necessary to inject the test dose intravenously at a rate not less than 1 to 3 cc per minute. Injections of these volumes may be given readily via the dorsal penile vein as this route permits the solution to be introduced at any desired rate, avoiding anesthesia, surgery, or the hazard of cardiac damage. (In some exploratory experiments the duration of infusion via the penile vein was extended to thirty minutes or more, with volumes up to 25 cc.)

As mentioned when the material was given at rates of 1 to 3 cc or more per minute, fatal anaphylaxis resulted in some animals, and various degrees of shock symptoms were exhibited in nonfatal cases (Table I). If, however, the same amount of material was infused at 0.25 to 0.5 cc per minute the incidence of any symptoms was extremely low and fatalities never occurred.

It should be noted that although volumes ranging from 3 to 12 cc or more were given to different animals the onset of anaphylaxis as seen in retrospect invariably occurred with 3 cc or less if the injection rate remained constant. The larger amounts were given only to assure ourselves that the absence of anaphylactic symptoms was not due to insufficient material. Therefore in later experiments 3 cc of solution were uniformly employed.

When animals were tested after three to four weeks rather than after a full month, the percentage of reactions was diminished and the severity of those observed was less. From one group of twenty guinea pigs sensitized as usual, three animals were taken at random after twenty eight days of incubation and given a challenging dose of the antigenic hydrolysate. Their reactions ranged from negative to only minimal symptoms. Two others tested three days later died of anaphylactic shock and in subsequent tests two thirds of the animals exhibited moderate to severe reactions. This noticeable change in apparent sensitivity in three days might be coincidental. However, it seemed expedient to set an arbitrary thirty to thirty one day minimum incubation period, and subsequent results seemed to justify waiting the few additional days.

Some variations in sensitivity were encountered in different groups given the same antigenic material and certain animals (less than 20 per cent on the average) were relatively resistant to sensitization under any conditions, a variability noted repeatedly but commonly not so distinctly as with our antigenic material. For the most part however sensitivity could be established by the procedure already detailed and it could be maintained or even increased by the intraperitoneal administration of a booster dose. The same amount as the single dose described was given to any animals surviving a test procedure or remaining unused for a period of time. These animals could be used again for testing after eight to fourteen days. Some have been reused in this manner as often as twenty times with no significant decrease in sensitivity. Adequate sensitivity of a group was established each time by selecting representatives at random and challenging them with minimal amounts of material (0.5 to 1.5 cc) sufficient to elicit anaphylactic symptoms without causing death, so that these pilot individuals might be saved for later use.

Table I summarizes the results of the various sensitizing schedules attempted. It will be noted that most of them employ a total of 500 to 750 mg of material, whereas the successful technique just described exceeds 1 Gm total solids. The alum precipitation method (to be discussed later) also showed as successful results only when a similarly larger amount of total solids was used.

When guinea pigs were sensitized with solutions which had been hydrolyzed for longer times, the antigenic potency was progressively reduced, until a point was reached where sensitivity no longer could be demonstrated (see Table II). This had been shown earlier in dogs by Sayhun and co-workers<sup>8</sup> with an acid hydrolysate of casein. With the fibrin hydrolysate, more than half of the nitrogen was in the free amino acid form at this point, and the peptides still remaining apparently were essentially devoid of antigenic characteristics.

If, however, other materials of approximately the same degree of hydrolysis, which by themselves were not found to be capable of sensitizing animals, were then used as the challenging dose for animals sensitized with the known antigenic material mentioned, anaphylactic reactions were occasionally encountered. This might imply that the challenging material may contain substances which fail to produce antibodies, but which are capable of reacting with the antibodies of adequately sensitized animals. This interpretation is in accord with the findings of Landsteiner on the demonstration of anaphylaxis by resorcinol diazo dyes that are by themselves not able to induce sensitization.<sup>9</sup> On the other hand, it is possible that if the animals were given the material a sufficient number of times, antibodies might be formed, although such a result could not be demonstrated with as many as nine injections of such material.

With other similar materials it was sometimes possible to find no anaphylactic symptoms when the challenging dose of 3 to 6 c.c. was given at 2 to 3 c.c. per minute, yet when other animals of the same group received the same amount of material at 6 c.c. per minute, anaphylactic reactions were occasionally elicited.

Partial despeciation of the parent material was demonstrated repeatedly, as might be expected from such a hydrolytic procedure. Animals sensitized to a partial hydrolysate of beef fibrin would show anaphylactic reactions, ranging to fatal, when challenged with a partial hydrolysate of hog fibrin, and vice versa. However, some degree of specificity still remained, as was shown by sensitizing groups of animals with a mixture of beef and hog fibrin hydrolysates, each known to be antigenic. Occasionally, after challenging such animals with one of the sensitizing agents, and allowing for the disappearance of shock symptoms, the injection of the other agent would elicit a second anaphylactic response, whereas animals receiving a second injection of the original agent showed no further symptoms. We interpreted the latter observation as sufficient evidence to rule out incomplete reactions.

In all the experiments described, results were checked by the injection of the material under parallel conditions into nonsensitized animals. In no instance were any symptoms noted which in any way resembled the anaphylactic responses obtained in sensitized animals. Conversely, injection of nonantigenic

material into animals known to be sensitized to the antigenic hydrolysate likewise elicited no anaphylactic effects. In fact, with either procedure usually nothing more was noted than would occur with the injection of the same volume of physiologic saline given at a similar rate.

More recently, the technique of alum precipitation to enhance antigenicity has been tried. To each 100 c.c. of antigenic hydrolysate (a 5 per cent solution) 30 c.c. of 10 per cent aqueous potassium alum were added and sufficient NaOH was used to adjust the pH to 7.0. After thirty minutes with repeated agitation, the material was centrifuged. The supernatant solution was either employed to resuspend the precipitate or was discarded and more of the original hydrolysate solution was used instead. Earlier groups received the precipitate intraperitoneally (with or without supernatant fluid) from material equivalent to 750 Gm solids of parent hydrolysate. Later groups received an amount equivalent to 1 Gm or more. Results have varied with some groups showing a high proportion of sensitive animals while other groups had a low incidence of sensitization. As can be seen in Table I c, there appears to be some correlation with the amount of sensitizing material.

Despite the reasonably comparable incidence of anaphylactic reactions in the alum precipitate sensitized animals, the resulting palpable masses of unabsorbed material that remained in the abdominal cavities of the guinea pigs rendered this method less desirable for our purposes, particularly inasmuch as we wished to use the animals repeatedly over long periods of time. In a few animals multiple adhesions and other evidence of irritation were noted. In one there appeared to be a sterile necrosis. On the other hand, several hundred animals have received the six dose injections of unaltered hydrolysate and none have been observed to develop pathologic changes in the abdomen.

Separate tests on the precipitate (redissolved) and on the supernatant solution from the alum precipitate method on other sensitized animals revealed that antigenic properties were present in both.

It is probable that the antigen of such protein hydrolysates is not a single entity but its detection or isolation has not yet been accomplished. Saturated trichloroacetic acid precipitation tests are not sufficiently sensitive, as antigenicity exists when no precipitate is present. Ten per cent picric acid precipitation also fails to differentiate, as an antigenic solution may give either a positive or negative test for precipitate. Saturation with ammonium sulfate still fails to provide complete correlation since a material may show haze or precipitation by this test and yet not be demonstrably antigenic. However in our limited experience in no case was it possible to demonstrate antigenicity when haze or precipitation was absent.

#### DISCUSSION

Many other individual experiments too numerous and scattered to describe here serve to corroborate further the observations reported. It is apparent that three factors, each necessary but not sufficient may be interdependent to determine the degree of sensitivity demonstrable with weakly antigenic protein

hydrolysates The incubation period was the first to be suspected, and extending it beyond four weeks brought about some increase in the incidence of shock symptoms The three-week period usually used with strong antigens was uniformly insufficient

The number of sensitizing injections is apparently important if the total mass of the antigenic moiety is a requisite in evoking antibody production From our results it is not possible to state definitely whether the antigenic hydrolysates contain a small amount of a potent antigen or a larger amount of a weak antigen However, in the experiments with the alum-precipitated material showing antigenicity both in the precipitate and supernatant solution, the impression would be that we are dealing possibly with multiple antigenic materials, all of which are weak

The speed of injection, when varied over a tenfold range, can be shown to have a significant influence on anaphylactic symptoms, whether the antigen be weak or strong, provided that the proper range is chosen This is probably a matter of controlling the rate of antigen-antibody combination with its release of histamine and/or other substances responsible for the anaphylactic symptoms When all factors are operating at their optimal level, sensitivity may be demonstrated more consistently, whereas a deficiency of any one of the factors will materially diminish the incidence of shock reactions

The evidence accumulated also emphasizes that the use of the converse procedure—namely, sensitizing with a hydrolysate to be tested for antigenicity and shocking with the known antigenic lot of digest—presents two possible shortcomings The first is that some lots tried, as mentioned earlier, failed to sensitize animals and yet would produce shock in animals sensitized with the antigenic hydrolysate This means that antigenic potentialities might be missed by this method The second disadvantage is merely one of time, since the presence or absence of antigenic characteristics would not be known for at least six weeks, while the use of the technique described herein at length affords an immediate answer when a stock of animals sensitized to the antigenic product is continuously available

#### SUMMARY

With weakly antigenic protein hydrolysates, at least three factors are important to insure satisfactory demonstration of anaphylaxis in guinea pigs These are a larger number of sensitizing injections, providing a greater total amount of material, a prolonged incubation period of at least one month, and a rate of intravenous injection of about 2 cc per minute

Antigenic substances produced from fibrin by acid hydrolysis may be partially despecciated and may not cross-react with the parent material They also are destroyed ultimately by further hydrolysis, before the breakdown into amino acids is complete

Related materials from different species may have both common antigens and at the same time specific antigens

The authors wish to acknowledge with appreciation the suggestions and constructive criticism received from Dr M W Chase during the course of these experiments

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## STUDIES OF AGGLUTINATION AND INHIBITION IN TWO LEWIS ANTIBODIES

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LEWIS antibodies originally were described by Mourant,<sup>1</sup> who found two O serums containing an identical antibody which agglutinated a previously unknown blood type. Both serums originated from women having boine children suspected of having erythroblastosis fetalis. In one case serum from the mother agglutinated cells from the child, whereas in the other case no agglutination occurred. In one case the woman's serum agglutinated cells from the husband, M<sub>1</sub> Lewis, thus the name. In a series of ninety-six O blood specimens the Lewis agglutination was independent of the Rh, MN, P, Lutheran, and Kell types. Of the ninety-six O blood specimens, twenty-four were Lewis positive, one doubtful positive. A study of fifteen families showed that the Lewis type probably was a dominant hereditary quality, though this was not statistically proved.

On the other hand Andriessen<sup>2</sup> has studied the Lewis agglutination in 500 adults of which 21 per cent were Lewis positive. Testing of children showed that about 70 per cent of the infants were Lewis positive before the age of 6 months. The incidence decreased with age to about 21 per cent as found in adults, and at the age of 1 year was about the same as in adults. According to Andriessen the Lewis types of mothers and their children strongly indicated that the 21 per cent Lewis-positive adults represented homozygotes of a possible Lewis-positive gene ( $L/L'$ ), furthermore that the about 70 per cent Lewis-positive infants represented homozygotes and heterozygotes ( $L/L' + L'/L'$ ), and that  $L'$  would become dominant to  $L'$  with increasing age.

### MATERIALS

The present investigations are mainly based upon two serums, one of which (An) by Andriessen has been characterized as identical with his own anti-Lewis serum, but weaker, and by Mourant as a potent Lewis antibody. The serum An originates from an A<sub>1</sub>MN 11 patient, a bipara whose children were healthy at birth. The cells of the actual child, type A MN 11, Coombs negative, were not agglutinated by serum from the mother by any of the methods mentioned below.

The other serum, Ness, originates from an A<sub>1</sub>M R<sub>1</sub>R<sub>2</sub> patient, a bipara whose children were healthy at birth. The husband was O MN R<sub>1</sub>R<sub>2</sub>. His cells behaved in serum Ness and serum An as a slightly Lewis positive.

Serum An had an anti-B titer of 2048; cold agglutinin titer of 2, Lewis antibody titer of 2 in saline medium, 8 in serum medium.

Serum Ness had an anti-B titer of 1024, cold agglutinin titer of 4, Lewis antibody titer of 4 in saline medium, 8 in serum medium.

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Both anti B titers were higher than the average the cold agglutinin titers were average values in our hands.

The Lewis antibody titers have been checked microscopically by Rh technique against a 2 per cent suspension of Lewis positive cells in saline and in serum medium respectively (serum from a Lewis negative person). The agglutination was only slightly stronger at room temperature than at 37° C. Both antibodies gave negative reactions by Coombs' test. Poor and unreliable reactions were obtained when albumin medium was used.

*Relationship of the Two Serums to O and H Antibodies*—When the two serums were tested at +5° C. a panagglutination was seen due to the cold agglutinins. It could not be demonstrated that this agglutination was stronger in A<sub>2</sub> and O blood specimens compared with A<sub>1</sub> blood specimens. Neither after absorption of the serums with A<sub>1</sub>B cells could any cold agglutination against A and O cells be demonstrated.

After Morgan<sup>3</sup> the formerly named anti O reagents may be subdivided into two groups:

1 The group of H antibodies, consisting of the heteroantibodies Shigella goat immune and cattle anti O reagents and an unknown proportion of human anti O reagents. The H antibodies are strongly active on O, A and AB cells, weaker and unreliably active on B, A<sub>1</sub> and A<sub>1</sub>B cells. These antibodies are easily inhibited in human saliva independent of ABO genotype.

2 The group of O antibodies of human origin, which agglutinate a product of the O gene in the cells. These antibodies are not inhibited by saliva.

Four anti O reagents found in our laboratory in serums from pregnant women have according to this very markedly different behaviour in saliva inhibition tests been subdivided into two most probably H antibodies and two most probably O antibodies. These antibodies agglutinate cells from Ness in the following manner:

Two O antibodies nonabsorbed	+
Two O antibodies absorbed with A <sub>1</sub> B cells	+
Two H antibodies nonabsorbed	(+)
Two H antibodies absorbed with A <sub>1</sub> B cells	—

Since cells from Ness are agglutinated by O and H antibodies such anti bodies cannot be present in her serum.

Cells from An were not available for these tests.

A<sub>1</sub>A<sub>2</sub> subdivision in the material below is performed with a potent anti A<sub>1</sub>, produced from an anti A serum by absorption with A cells. The Rh types are tested with Rh antibodies not containing Lewis antibodies.

#### METHODS

Besides the definitely positive reactions doubtful reactions always were encountered in serum An at 37° C. and at room temperature by all methods employed (cells suspended in serum, citrated plasma, saline, dextrose saline). Because of this fact, the following technique was used in a more extensive series. Cells from O and A clots (blood donors) about 24 hours old were suspended to about 1 per cent in saline, centrifuged, and resuspended to about

1 per cent in saline. One drop of the cell suspension and 1 drop of serum An (anti-B not absorbed) were mixed in a Rh tube, incubated for two hours at 37° C, and read microscopically on slides. By this technique doubtful reactions generally were avoided.

In a series of 285 nonrelated blood donors whose A<sub>1</sub>, A<sub>2</sub>, O, and sex distribution were within normal limits, 21.75 per cent Lewis positive were found by this technique, independent of MN and Rh type, and in a smaller group also independent of P type. The agglutination, however, was not completely independent of A<sub>1</sub>, A<sub>2</sub>, and O type, as shown in Table I.

TABLE I

		NUMBER INVESTIGATED
A <sub>2</sub>	27.7% Lewis positive	36
A <sub>1</sub>	22.9% Lewis positive	109
O	19.3% Lewis positive	140
Total	21.75% Lewis positive	285

However, other techniques gave a higher frequency of Lewis positives. A series was tested at 37° C in parallel with two different concentrations of cells: 1 per cent washed suspension and 5 per cent unwashed suspension of cells. One drop of each concentration was incubated for two hours with 1 drop serum An and then read microscopically, any trace of agglutination being recorded as positive. The results obtained are shown in Table II.

TABLE II

NUMBER	1% SUSPENSION	5% SUSPENSION	
10	+	+	Termed strongly Lewis positive
8	-	(+)	Termed slightly Lewis positive
29	-	-	Termed Lewis negative

The test gave the same results in cells from fresh etiated blood as in cells from clotted blood stored for twenty-four hours. The same technique used at room temperature on the same series added some doubtful agglutinations in the 5 per cent suspension, but not in the 1 per cent washed suspension. In slightly Lewis-positive blood specimens also Coombs' test was negative. Absorption series consisting of a total of forty samples gave the results shown in Table III.

TABLE III

	SERUM AN ABSORBED AT ROOM TEMPERATURE WITH 1/5 VOL. CELLS OF TYPE		
	STRONGLY LEWIS POSITIVE	SLIGHTLY LEWIS POSITIVE	LEWIS NEGATIVE
Absorbed serum tested at room temperature against 5% suspension of			
Strongly Lewis positive cells	-	+	+
Slightly Lewis positive cells	-	-	(+)

Even serums absorbed with equal volume of slightly Lewis-positive cells agglutinated strongly Lewis-positive cells. However, absorption tests with 1/5 volume of cells were not quite reliable as some of the strongly Lewis-positive blood specimens did not cause a complete absorption. The serums absorbed may

agglutinate slightly when tested against other strongly Lewis positive blood specimens

*Action on Cells From the Venborn (Blood Specimens From Umbilical Cords)*—Our anti Lewis serums have been found to agglutinate these blood specimens, but very slightly compared with blood from adults which is confirmed by Andresen<sup>4</sup>

For a closer examination of serum An inhibition tests in saliva were attempted. However serum An obviously readily deteriorated in saline. Serum Ness was stable in saline. Serum An and serum Ness behaved in the same way at 37° C, but not when a 5 per cent suspension of cells was used at room temperature. Serum Ness agglutinated weakly more samples than serum An at room temperature.

*Inhibition Technique With Serum Ness*—Samples of saliva rapidly collected, were boiled for twenty five minutes, centrifugated and some of them stored frozen for some days. The saliva was diluted with saline (volumes of 0.2/1.8 c.c.) as shown in the following series:

1     $\frac{1}{10}$      $\frac{1}{100}$      $\frac{1}{1000}$      $\frac{1}{10000}$

One drop of each of these dilutions was mixed with 3 drops of serum Ness (anti B not absorbed) in Rh tubes and incubated for ten minutes at room temperature. Two drops of each of these mixtures were then transferred to slides and mixed with 1 drop of a 1 per cent suspension of strongly Lewis positive cells in saline. The slides were placed in a moist chamber at room temperature and read after thirty and sixty minutes. The following were used as controls: a 3 drops serum/1 drop saline mixture and a 3 drops serum/1 drop saliva mixture (undiluted saliva from female Lewis negative Lewis antibody producers). The number of positive reactions was not larger after sixty minutes than after thirty minutes.

TABLE IV. AGGLUTINATION INHIBITION TESTS IN SERUM NESS WITH SALIVA FROM 49 PERSONS WHOSE CELLS ARE CLASSIFIED BY MEANS OF SERUM NESS AS STRONGLY LEWIS POSITIVE (LEWIS +, 14 INDIVIDUALS), SLIGHTLY LEWIS POSITIVE (LEWIS +), 3 INDIVIDUALS) AND LEWIS NEGATIVE (12 INDIVIDUALS)

SALIVA DILUTIONS		SALIVA FROM		
NO INHIBITION		LEWIS + PERSONS	LEWIS (+) PERSONS	LEWIS NEG PERSONS
1/10000	1/1000	3		
1/1000	1/100	9		3
1/100	1/10	2	2	11
1/10	Undiluted		1	10
No inhibition in undiluted saliva				8

Three Lewis antibody producers

Lewis group specific inhibition in serums was investigated in fifty blood donors. The saliva inhibition had been studied in most of these cases. One drop of the serum to be investigated was mixed with 1 drop of serum Ness in an Rh tube, incubated for ten to fifteen minutes at room temperature, transferred to slides, and tested as in the saliva inhibition test. By this method the agglutination was inhibited only in serums from strongly Lewis positive individuals.

The serum inhibition was then titrated in the following manner. To a series containing increasing amounts of serum Ness in Rh tubes, 1 drop, 2 drops, 3 drops and so on, was added 1 drop of the serum to be investigated. The same technique as outlined was used otherwise. Agglutination (no inhibition) varied from one case to another from 3 to 7 drops of serum Ness. As an average, inhibition occurred by 3 volumes of antibody being added to 1 volume of serum, whereas 4 volumes of antibody to 1 volume of serum caused agglutination. There was no marked parallelism between saliva and serum inhibition in the same individuals.

*Relation to the B Group*—After absorption of anti-B in serum Ness with B Lewis-negative cells, three of sixteen B blood specimens were found Lewis positive, thirteen Lewis negative.

*Relation Between Cold Agglutination and Lewis Type*—Serums obtained from ten donors containing cold agglutinins of medium strength were tested against panels of fifteen blood specimens of known Lewis type by the following technique. 1 drop of a 3 per cent suspension of cells was mixed with 2 drops of serum on slides and incubated at +5° C for fifteen to thirty minutes. No relationship could be traced between the Lewis type and the intensity of the cold agglutination.

#### COMMENTS

It is evident that the saliva of most persons termed Lewis negative contains Lewis-positive substances, which inhibit serum Ness. The simplest explanation to this may be as follows. Estimated by serum Ness, the erythrocytes of most individuals termed Lewis negative contain too small amounts of Lewis-positive substances to be traced by serum Ness. On the other hand, sufficient amounts of Lewis-positive substances will be present in the secretions of the same individuals. The phenomenon that inhibiting Lewis-positive substances can be traced in saline used for washing of strongly Lewis-positive cells, but not in saline used for washing of Lewis-negative cells from Lewis-positive secretors, can be explained in the same way.

The deviation in A<sub>2</sub>, A<sub>1</sub>, O distribution as to the incidence of strongly Lewis-positive individuals is statistically nonsignificant.

#### SUMMARY

Two Lewis antibodies are studied. Among 285 individuals, 21.75 per cent were found Lewis positive. Inhibition phenomena in serums and saliva from about fifty individuals are examined. In serum inhibition tests, only Lewis-positive individuals presented a Lewis group specific inhibition in their serums. In saliva inhibition tests, forty-one of forty-nine saliva specimens presented a Lewis group specific inhibition.

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## THE OCCURRENCE OF ALSO POSITIVE TRICHINA PRECIPITIN TESTS IN INFECTIOUS MONONUCLEOSIS

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THE finding of a positive trichina precipitin test is not in itself sufficient to justify a diagnosis of trichinosis but the fact that it often influences the observer in favor of this diagnosis cannot be denied. It may generally be stated that when the test is made there is some clinical or laboratory evidence that makes this disease suspect. For this reason a positive report may be considered, in addition to other findings sufficient to justify a diagnosis of trichinosis without resorting to further tests. In the final analysis however only a positive muscle biopsy justifies a positive diagnosis. It seems important therefore to report that in infectious mononucleosis a disease notorious for its protean clinical manifestations the trichina precipitin test has been found positive in fairly high titers in several instances. In all these the test was ordered because for one reason or another trichinosis was considered as a possible diagnosis. The first two cases which are reported in detail indicate how easy it is to be misled when undue emphasis is placed on this test.

### CASE 1—

*History*—A H, a 21 year old student nurse was admitted to the Nurses Infirmary on Feb. 18, 1947 because of sore throat and malaise. Her temperature was 100.4 and the physical findings were limited to slight reddening of the pharynx and several palpable small anterior cervical glands. The spleen was not palpable and there was no rash. A provisional diagnosis of upper respiratory infection and possible infectious mononucleosis was made. The first blood count was reported as follows: hemoglobin 81 per cent, red blood cells 4,700,000, white blood cells 12,800, neutrophils nonsegmented 12 per cent, segmented 44 per cent, lymphocyte 28 per cent, monocytes 8 per cent, basophiles 3 per cent, eosinophiles 7 per cent. There were no abnormal lymphocytes suggestive of infectious mononucleosis. Because of the eosinophilia, the count was repeated the next day and revealed 11 per cent eosinophiles. Further questioning revealed that the patient had eaten pork on several occasions within the preceding three weeks. There was no history of allergy. She had had scarlet fever at the age of 4 and rheumatic fever at the age of 9 without cardiac sequelae. At 16, the patient had pneumonia followed by empyema which did not require surgical drainage. She had lost about 15 pounds in the past three months and during this time she thought her eyelids were somewhat puffy on arising in the morning. A skin test with trichinella antigen was performed the second day and was negative. Stool examinations were repeatedly negative for ova and parasites. On the third day in the Infirmary the patient had a shaking chill with a sharp rise in temperature to 103 associated with severe muscular pains, and was admitted to the hospital.

*Physical Examination*—The patient appeared neutely ill. There were a short hacking cough, a profuse postnasal drip and a severely stuffed nose. The palpable anterior and posterior cervical glands noted on admission to the Infirmary were significantly increased in size. The heart and lungs were normal. The abdomen was soft. The spleen was not palpable. There was marked tenderness of the calf but no other evidence to suggest thrombophlebitis. No physical findings suggesting urinary tract infection were elicited.

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*Laboratory Data*—X ray of the chest was negative X rays of the sinuses revealed a slight increase in the density of the frontal and right ethmoid sinuses Urine examinations were repeatedly negative except for the presence of acetone on two occasions when the patient was severely dehydrated after prolonged vomiting Blood counts were taken at frequent intervals and persistently revealed an eosinophilia Table I indicates the course of the shift in the white cells toward the lymphoid side Urine and blood cultures were negative While still in the Infirmary, before she became acutely ill, a specimen of blood had been sent to the Bureau of Laboratories of the New York City Department of Health for a precipitin test for trichinosis This was reported as positive in a dilution of 1 640 The test was repeated a week later and reported "serum excellent, test positive, dilution 1 640, clear cut "

TABLE I CASE 1

DATE	WHITE BLOOD CELLS	SEG	STAFF	LYMPH OCYTES	EOSINO PHILES	MONOCYTES
Feb 18	12,800	47	12	26	7	8
19	8,700	49	4	26	11	10
22	6,900	48	7	31	8	6
24	6,450	25	15	39	10	11
25	7,400	46	14	27	5	8
26	10,350	29	39	24	2	6
27	9,400	46	12	28	5	9
28	10,750	36	16	42	0	5
Mar 1	7,800	35	11	44	0	10
3	8,450	41	7	37	1	14
4	11,500	36	12	43	1	8
5	11,300	20	13	59	1	7
<i>(Cells typical of infectious mononucleosis found)</i>						
6	8,100	21	13	66	0	0
7	9,550	18	11	70	0	1
8	13,500	16	6	72	0	6
10	16,700	7	6	86	0	1
11	18,800	17	7	70	0	6
12	12,300	19	2	78	0	1
13	15,800	21	1	78	0	0
14	13,000	22	2	66	0	10
15	12,400	35	3	56	0	6
17	10,300	28	5	60	0	7
18	10,800	36	1	59	1	3
May 26	11,100	55	0	33	7	5

*Course*—With the history of pork ingestion, fever, severe muscle pains, eosinophilia, and a positive precipitin test, it was felt that this was a case of trichinosis The patient ran a very stormy course for six days with temperature fluctuating between 100° and 104° During the second week the temperature slowly began to subside and reached normal during the third week where it remained to the time of discharge, five weeks after admission During the height of the febrile period, the patient complained of marked anorexia, occasional vomiting, and severe muscle pains On March 6, two weeks after acute onset, as the temperature was approaching normal, the peripheral blood for the first time revealed abnormal lymphocytes typical of infectious mononucleosis, and the heterophile reaction was reported positive in a dilution of 1 400 Another specimen of blood was sent to the Department of Health Laboratories and this precipitin test was negative for trichinosis Repeat heterophile reactions were reported as positive in dilutions of 1 400, and the peripheral blood showed abnormal lymphocytes on several occasions The patient's general condition rapidly improved after the third week and she was discharged in good condition Two months later, the heterophile was weakly positive 1 100, and the blood count still revealed 7 per cent eosinophiles

*Comment*—This case in retrospect, seems fairly typical of infectious mononucleosis. The rather high eosinophile counts however, sidetracked us and the positive trichina precipitin tests tended to lead us further astray. These findings and the additional corroborative evidence of pork ingestion spoke strongly for a diagnosis of trichinosis. The appearance however, of the typical blood picture for infectious mononucleosis two weeks after the onset threw doubt on the validity of the positive trichina precipitin test. The strongly positive heterophile agglutination reaction left no doubt as to the correct diagnosis, but left the positive precipitin tests unexplained. Accordingly, the third test was made and was reported negative.

CASE 2—G. G. was a 33 year old white man who consulted his physician April 1, 1948, because of severe pains in the chest back and neck. The pains were considered at the time as being muscular in origin. The physical examination was essentially negative. He was seen again four days later at which time he had fever, sore throat and chilly sensations. The physical examination was still completely negative. Because the fever could not be explained, blood was taken for the routine agglutination tests and a trichina precipitin test was done because the pain in the chest was considered to be at the site of the insertions of the diaphragm and to be muscular in origin. Three days later all tests were reported negative except the trichina precipitin test which was positive in a dilution of 1:1280. The heterophile antibody titer was 1:14. On April 10 a blood count was done to determine the presence of eosinophilia, and was reported as follows: white blood cells, 9,000, lymphocytes 66 per cent. No eosinophiles were present. The picture was reported as being suggestive of infectious mononucleosis. On April 12 a complete count was reported as follows: hemoglobin, 92 per cent, red blood cells 4,650,000, white blood cells, 9,300, neutrophils nonsegmented, 14 per cent, segmented, 15 per cent, lymphocytes 58 per cent, atypical lymphocytes, 9 per cent, monocytes, 4 per cent. These counts were considered quite characteristic of infectious mononucleosis. As a result the heterophile agglutination and trichina precipitin tests were repeated. On April 17 the heterophile was reported strongly positive in a dilution of 1:1,792. At the same time, the precipitin test was positive in the same titer as reported previously, 1:1,280. The same serum was sent to the National Health Institute at Bethesda, Md. for a complement fixation test for trichinosis. This was reported positive. A skin test done at the same time was negative.

On April 16 the white count was 24,000 with 80 per cent lymphocytes, many of which were atypical. A sternal bone marrow aspiration was reported as showing no evidence of leukemia. On this date the patient appeared and felt quite well. At no time during the illness was there an adenopathy or splenomegaly. Trichinosis was considered briefly as the diagnosis and the positive precipitin test proved quite misleading. However, the early development of the characteristic blood picture indicated the true diagnosis. The patient was not seen again until June 2, 1948 at which time the heterophile agglutination test was still positive in a high titer but the trichina precipitin test was completely negative. He appeared and felt perfectly well.

CASE 3—Mrs. R. was a 34 year old white medical research worker who on Feb. 1, 1947, began to experience a sore throat, general malaise, and muscle pains. There was no adenopathy or enlarged spleen. No blood studies were made, but on Feb. 20 the lymphocyte count was reported as 60 per cent. There was no note of a more detailed count. On Feb. 24 the heterophile agglutination test was reported positive as was also the trichinosis precipitin test. The precipitin test was negative on March 7, but the heterophile was still positive. The patient made a rapid, uneventful recovery.

CASE 4—L. Z. was a 19 year old, white stenographer who was admitted to the wards of Long Island Medical College, Feb. 20, 1947. She gave a history of fatigue and sore throat. The throat had a whitish membrane. She received diphtheria antitoxin and penicillin. She

appeared acutely ill and had a temperature of  $104.0^{\circ}$ . There was generalized lymphadenopathy, but the spleen was not palpable. The blood count on admission revealed a white count of 8,700, polys, 66 per cent, eosinophiles, 6 per cent, lymphocytes, 22 per cent, monocytes, 5 per cent, plasma, 1 per cent. Two days later the lymphocytes were 42 per cent, with many atypical forms. The eosinophilia remained at 6 per cent. The heterophile was positive at this time, as was also the precipitin test. The titers and dates of tests may be seen in Table II. The patient was discharged March 6, 1947, quite well. There seems no doubt that the patient had infectious mononucleosis. It might be mentioned here that she was Jewish and observed the dietary laws. To her knowledge she had never in her life eaten pork.

TABLE II

DATE	CASE	HETEROPHILE	PRECIPITIN
2/18/47	1		1:640
2/25/47	1		1:640
3/ 6/47	1	1:400	Neg
4/ 8/48	2	14:140	1:280
4/15/48	2	1,792:896.0	1:280
6/ 1/48	2	896:448.0	Neg
2/24/47	3	224:112.0	640
3/ 7/47	3	112:56.0	Neg
2/21/47	4	448:224.28	1:280
2/27/47	4	896:448.0	1:280
3/ 4/47	4	896:448.0	640 (faint)
4/11/47	5	896:448.0	1:280

All heterophile and trichina precipitin tests with the exception of the heterophiles in the first case were performed by the New York City Department of Health.

The three figures in the heterophile reports indicate unabsorbed serum, guinea pig kidney absorbed and beef red cells absorbed.

CASE 5—M. C. was a white, 21 year old, male machinist who was admitted to the wards of the Long Island Medical College March 30, 1947. He gave a history of shaking chills ten days before admission. He had general malaise, muscle pains, and sore throat. He was given penicillin without effect. He appeared only moderately ill, but had a temperature of  $103^{\circ}$  on admission. There were enlarged posterior cervical glands and the spleen was one finger breadth below the costal margin. The white blood count was 5,900, with 46 per cent lymphocytes. Lymphocyte percentage rises went to 60 per cent with many atypical forms. On April 11 a heterophile agglutination test and a trichina precipitin test were done. Both were reported positive in high titers. The patient was discharged April 19 feeling perfectly well, with a final diagnosis of infectious mononucleosis. Unfortunately, no further serologic studies were made.

#### DISCUSSION

Beinstein,<sup>1</sup> in his very complete monograph of infectious mononucleosis, discusses false serum reactions in this disease. He does not, however, mention the finding of positive trichina precipitin tests in any of his cases. A review of the literature since this monograph revealed no report of this unusual finding. Bernstein pointed out that the serum substances giving false positive Wassermann reactions are probably in no way associated with the sheep cell agglutination reaction. In his own series of cases, he found that the false positive tests for syphilis often appeared before the heterophile reactions became positive. Thus dissociation appears also to be true in some of our cases where the precipitin tests became negative when the heterophile antibody titers were still high.

The trichina precipitin test as performed in the Bureau of Laboratories of the New York City Health Department follows the dicta laid down by the



National Institute of Health, Bethesda Md. The antigen is carefully prepared and subjected to a long process of purification. The sera to be examined are cleared of all turbidity by rapid centrifugation in a refrigerated centrifuge. Dilutions from 1:80 to 1:1280 are set up, and readings of 1:160 and up are considered presumptive evidence of trichinosis provided there is a clinical history, recent or dating up to three years back. Readings of course, may go very much higher. The value of this test is the subject of much controversy. Augustine considered it remarkably specific and had false positive reactions only when patients were taking quinine or arsenicals. It is fairly well recognized now however that false positive reactions may occur, as in periarteritis, and it has been noted to be positive at times in parasitic infections other than trichinosis. Recent reports by Fisch and co-workers<sup>3</sup> and Roth<sup>4</sup> indicate that the test is of little value and even when positive is too late to be of much diagnostic help. It is generally true that the test does not become positive until about four weeks after infestation and this should be borne in mind, but in severe cases it may on occasion become positive in a matter of days.

It is not the purpose of this report to laud or condemn the test. In its defense one might state that positive Wassermann tests occur in conditions other than syphilis but no one would suggest the test be discarded. It is essential however, that the lack of specificity of any test be known by those requesting it. Regardless of agreements and disagreements the fact remains that the trichina precipitin test is still used extensively, and to most physicians a positive report carries a good deal of weight. Skin tests are considered no more reliable and a muscle biopsy is not always feasible. It might be mentioned here that a positive biopsy may confirm the diagnosis but a negative report is not proof that the patient does not have trichinosis. The complement fixation test may be valuable but the positive report in our second case indicated that it too may prove misleading.

The blood count in trichinosis is of considerable importance. There is usually a leucocytosis and a marked eosinophilia.<sup>5</sup> However, on occasion the white count may not be elevated and in very severe cases there may be a complete absence of eosinophiles. In infectious mononucleosis the blood picture is considered to be of paramount importance. Nevertheless, the characteristic picture may not be present at the onset, and eosinophilia of a fairly marked degree has been reported.<sup>6</sup> Both of these unusual features were noted in Case 1, and to a lesser extent in Case 4.

In Cases 3, 4, and 5, trichinosis was not very seriously considered, but the positive precipitin tests proved temporarily misleading. In Cases 1 and 2 the positive tests were considered in addition to other features sufficient evidence to justify diagnoses of trichinosis. It may well be pointed out that the clinical features in these cases were not very suggestive of trichinosis, but students of this disease stress the fact that the clinical course varies greatly and indicate that the diagnosis is frequently missed because clinicians too often expect a classical history.

There would seem to be no doubt that the patients herein reported had infectious mononucleosis. In all, the blood pictures were ultimately confirmatory and in all, the heterophile agglutination tests were strongly positive. The one thing that tended to confuse in all instances was the occurrence of the positive precipitin tests. A possible explanation would be that these patients all had trichinosis whether recent or remote. This has to be given serious consideration in view of the fact that a high percentage of the population is thought to be infected. Even if the infection is not recent, it is possible to conceive of an anamnestic reaction boosting the precipitin titer. However, the very rapid return to normal in three of the cases makes this seem very unlikely, and in one (Case 4), if we may accept the history, the patient never had eaten pork in her entire life.

These patients were not found to have positive heterophile antibody agglutination tests and positive trichina precipitin tests as part of a planned study. The tests were requested by physicians before definite diagnoses were made and the findings as reported were found. The percentage of false positive trichina precipitin tests (or complement fixation tests) occurring in infectious mononucleosis is not known and would require an extensive study, but this small group indicates it would not be negligible.

Most physicians today are conditioned to the vagaries of this disease and have learned to give it due consideration in the differential diagnosis of any unexplained fever. The cases in this report stress the fact that even when it is considered, variations in the blood picture and false positive serological reactions tend to mislead. It has become more and more apparent that where the disease is once suspected, the diagnosis should be clung to tenaciously until completely ruled out or made with certainty.

#### SUMMARY

Positive trichina precipitin tests are reported in five cases of infectious mononucleosis. The evidence at hand indicates that these reactions were false positive reactions and were induced by the serum changes of infectious mononucleosis.

For permission to publish Case 2 we wish to thank Dr. J. Jerome Steinfeld and Dr. Samuel Weinbaum, New York, for Case 3, Dr. Marsh McCall, New York, for Cases 4 and 5, The Medical Department of the Long Island Medical College, New York.

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## THE CHRONIC TYPHOID CARRIER II THE EFFECT OF CHOLECYSTECTOMY ON THE BACTERIOLOGIC COURSE

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### INTRODUCTION

IN A previous paper<sup>1</sup> we have discussed the chronometry of the natural course of the typhoid carrier state. Because of the essential role of the carrier in the dissemination of typhoid and the infrequency of spontaneous cure of the carrier state, treatment is an important public health problem.

To date there is no effective nonsurgical therapy. In 1907 Dehler<sup>2</sup> reported the treatment of two carriers by cholecystostomy. Gummie<sup>3</sup> in 1908 described the cure of one patient by cholecystectomy. Thereafter many reports on the surgical treatment of carriers have appeared. In 1930 Roesler<sup>4</sup> compiled a very complete bibliography. Eichhoff<sup>5</sup> reviewed chiefly the European literature until 1930 and collected 102 cases in which gall bladder surgery had been employed in the treatment of the chronic typhoid carrier state. Because it was well known that the stools of some carriers do not clear up until several months after operation, he arbitrarily chose six months as a minimum follow up period for evaluation. Discarding eight cases for this reason, he found that seventy six, or 81 per cent, of the ninety four subjects were cured by operation.

Coller and Forsbeck<sup>6</sup> added eighteen cases to fifty five cases from the literature (chiefly from the United States) and reported 76.9 per cent cures for a combined total of sixty five patients surviving cholecystectomy.

The results of cholecystectomy are thus quite favorable. We have found, however, that only a few cases have been followed for a number of years after operation, and that there has been no evaluation of 'immunity' to reinfection afterward.

### THE CLINICAL MATERIAL

Of the fifteen typhoid carriers subjected to cholecystectomy eleven apparently had become infected during or after the 1939 Manteno epidemic previously described.<sup>1, 7, 8</sup> The remaining four were discovered in other state institutions and had been sent to Manteno for care. There were four male patients and eleven female patients, living in separate halves of the same isolation cottage. The dining room and kitchen facilities were shared, however. Both pre and postoperatively these patients lived with all the other typhoid carriers and were thus constantly being exposed to reinfection. This is emphasized in view of the postoperative bacteriologic course to be described.

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We are indebted to Dr. Otto Lohman, Public Health Officer at Manteno State Hospital, for the preparation of many of the bacteriologic records.

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*Preoperative Bacteriologic Status*—The average time between the date on which each patient was declared a chronic carrier until the date of operation was twenty-seven months ( $SD \pm 13$ ). In three subjects the duration was five, eight, and ten months, while in the remaining twelve the range was twenty to fifty-six months, averaging thirty-one months. In three instances in which the earlier state followed a diagnosed acute attack of typhoid fever, six to twelve

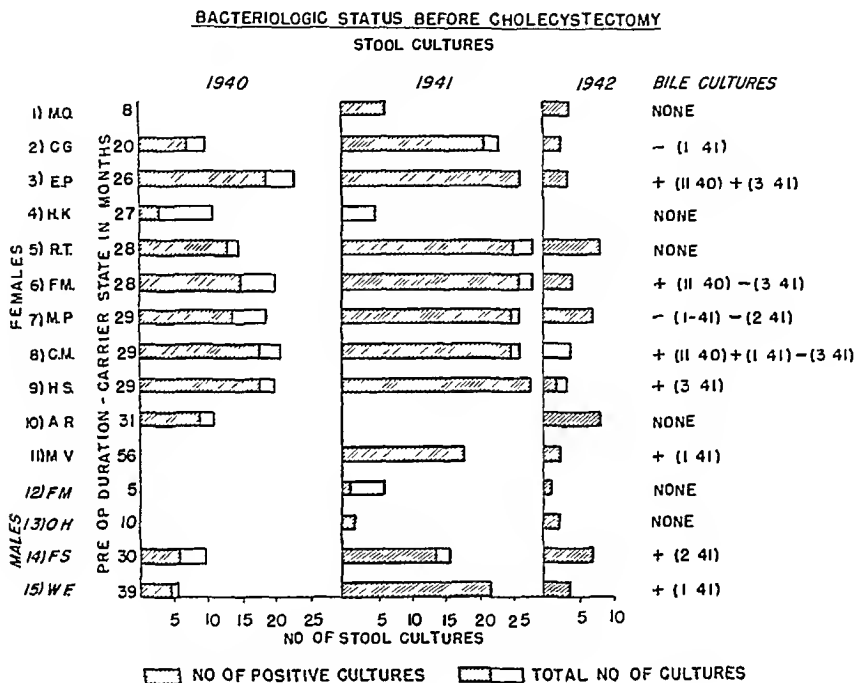


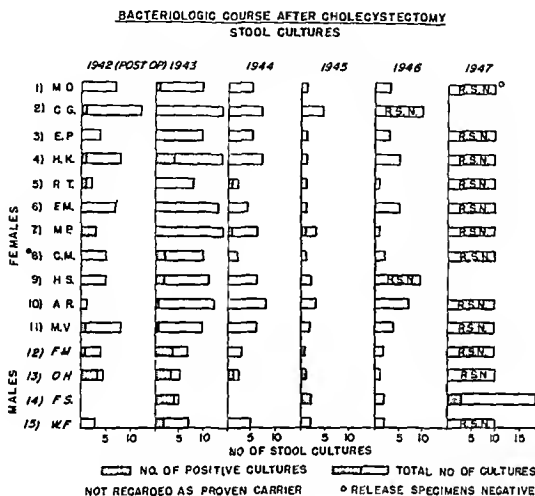
Fig 1

months elapsed between the onset of the attack and the designation as a chronic carrier. The remaining patients were designated as chronic carriers on the dates on which cultures positive for typhoid organisms were confirmed. In these patients there had been no clinical diagnosis of typhoid fever.

The preoperative bacteriologic studies on each patient are summarized in Fig 1. Each horizontal bar represents the total number of stool cultures for each subject for the year specified. The shaded portion of each bar shows the number of such specimens from which typhoid bacilli were isolated. These data are adequate to show that thirteen patients were definite chronic carriers and were excreting *Escherichia typhosa* at the time of cholecystectomy. In one patient (H K) it appears probable and in another (C M) possible that spontaneous remission had taken place. These two patients are included in the study to compare their bacteriologic course with that of the definite carriers.

Fourteen satisfactory bile cultures were made in nine cases within the eighteen-month period preceding surgery. In seven patients, or 78 per cent,

one or more positive specimens were obtained, these patients may be considered to have been biliary carriers. In two patients (C G and M P), one and two negative cultures respectively were obtained, although both patients had consistently positive fecal specimens. On the basis of so few bile samples, we considered the question of biliary infection in these two cases to have been unanswered.



Fig

The cholecystectomies were performed by Dr. Arnold Schumberg between January and May in 1942. The patients were transferred from the typhoid cottage to the surgical ward a day or two before operation. As soon as the patients were ambulatory after surgery, usually within two weeks, they were returned to their previous living quarters.

#### POSTOPERATIVE BACTERIOLOGIC COURSE

Using the same descriptive scheme as in Fig. 1, the postoperative bacteriologic findings are summarized in Fig. 2.

*Duration of the Carrier State After Operation*—In ten cases one duodenal aspiration was made on the third to the sixth postoperative day and a bile culture taken. Typhoid bacilli were found in nine specimens. The single negative report occurred in case 9, on the fourth postoperative day. In this patient a negative stool culture had been obtained three days before operation, but there had been thirty positive cultures, including one of bile, during the preceding year. The first stool cultures made after surgery were negative in nine of the

thirteen proved carriers. These were obtained at periods ranging from two days to ten weeks postoperatively, in seven instances between the third and eighth weeks.

In three additional cases the first stool cultures were positive, and the earliest persistently negative reports were not obtained until from ten weeks to 85 months after cholecystectomy.

In one case the stools remained intermittently positive until the end of this study.

*Pattern of "Recurrences"*—From Fig 2 it is seen that in only two cases (3 and 6) were there no positive cultures obtained after surgery. In seven additional cases, only one or two positive reports were recorded.

There is a definite diminution of the number of positive cultures with the passage of time. Although the data from 1944 and 1945 are not adequate in number, comparison of the findings in 1942-1943 and those in 1946-1947 shows a striking change. In 1946-1947 in all but one case the official criteria for release from designation as a carrier were fulfilled. These, in Illinois, consist of eight negative stool cultures at consecutive monthly intervals and two negative bile cultures seven days apart taken thirty days after the last stool culture.

*Sex Difference*—The small number of cases in this group does not permit a firm conclusion regarding the relation of sex difference to cure. However, of the 100 cultures made postoperatively in the male group, twenty-five were positive, while only fourteen, or four per cent, of the 329 cultures made in the female group were positive. This difference is statistically very significant ( $\chi^2 = 40$ ), and in Fig 2 it may be seen that there is a fairly even distribution of stool sampling throughout the five-year period. This indicates, regardless of the fewness of the cases, that a sex difference probably exists.

#### DISCUSSION

The observation that *bile cultures* were positive from three to six days after cholecystectomy in nine of the ten cases tested shows that the site of biliary tract infection is not the gall bladder alone. It is not likely that infected bile expelled from the gall bladder at the time of operation would itself remain in the biliary tract for the three to six-day period described. Nor could it be presumed that in each case an infected cystic duct stump had been left behind. It appears probable that the infected bile from the gall bladder contaminated extrahepatic structures or intestinal contents and that a week or longer was required to clear the contamination.

A sufficient number of stool cultures were not taken in the early postoperative period to provide precise data on the postoperative duration of the carrier state.

*It is a remarkable and hitherto undemonstrated fact that living amid constant exposure to reinfection from untreated carriers, twelve of thirteen proved typhoid carriers became and remained cured following cholecystectomy.* In a few instances prolonged reinfections appeared to have occurred. However, most of the positive cultures obtained postoperatively, especially in the female group, appeared sporadically and for such brief periods that we believe in some

instances there may have been excretion of the organisms as transients, without definite reinfection. The transient excretion of typhoid bacilli without evidence of actual infection has been shown to occur by Cler and Ferazzi.<sup>9</sup> They reported on the stool cultures of thirty-nine persons who had been known to eat the same infected food as others who developed clinical typhoid fever. Six of the asymptomatic individuals excreted typhoid bacilli for a period up to one week after the beginning of the disease in those who developed typhoid fever. We have observed such episodes of transitory excretion of typhoid bacilli in some carriers becoming spontaneously cured.<sup>1</sup>

In a previous report<sup>1</sup> we described the bacteriologic course of seventy-nine typhoid carriers living together with the patients reported in this paper. In these seventy-nine noncholecystectomized patients who served as ideal controls for the evaluation of therapy, 29 per cent of spontaneous cures occurred by the same criteria. In addition many long "recurrences" of the carrier state or lasting reinfections occurred after the carrier state had disappeared spontaneously for several weeks or months. These observations indicate that cholecystectomy was followed by a high degree of resistance to recurrence of the carrier state.

#### SUMMARY AND CONCLUSIONS

1 Cholecystectomy was performed on thirteen proved chronic typhoid carriers. Following operation these patients continued to live among many other typhoid carriers, some of whom were heavily infected. The period of study lasted five years after surgery.

2 Under these conditions of massive exposure to reinfection, twelve of the thirteen patients became and remained free of typhoid organisms.

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# LABORATORY METHODS

## AN APPRAISAL OF THE MALE NORTH AMERICAN FROG (*RANA PIPIENS*) PREGNANCY TEST WITH SUGGESTED MODIFICATIONS OF THE ORIGINAL TECHNIQUE

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### INTRODUCTION

ROBBINS and Parke<sup>1</sup> and Wiltberger and Miller<sup>2</sup> have recently described a new test for pregnancy employing as a test animal the male North American frog (*Rana pipiens*). In an attempt to evaluate the accuracy and efficiency of this new technique, the author tested 200 urine specimens from women suspected of being pregnant. Since the urine pregnancy test using the African female frog (*Xenopus laevis*) is an accepted, reliable procedure, a male *R. pipiens* and a female *X. laevis* were tested with each urine specimen, and the results compared. In addition, the advantages of Scott's<sup>2</sup> method of urine concentration are described in preparing the inoculum for *R. pipiens*.

### PROCEDURE

All urine specimens delivered to the laboratory for pregnancy testing were injected into the frogs following the technique of Wiltberger and Miller. Five cubic centimeters of whole, unmodified urine were filtered and made slightly acid to litmus with HCl. This material was injected into the dorsal lymph sac of the animal. Of the first ten animals treated in this manner, only one survived longer than one half hour. This latter animal died at the end of two hours. The high mortality could not, it seemed, be due to the toxicity of an individual urine specimen, since each animal was injected with urine from a different woman. Believing that the volume of the injection was too great to be tolerated by the animal, an attempt was made to give the urine more slowly by injection in stages 2, 2, and 1 cc at five minute intervals. This method also was unsuccessful, and 1 cc doses at five minute intervals were attempted. The animals survived the first 3 cc, but the fourth dose was almost invariably fatal within a few minutes. It was, then, apparent that if the frogs were to survive long enough to give a valid test, a small volume injection containing enough hormone to stimulate a gonadal reaction was needed to overcome the difficulty.

A modification of the original Scott method<sup>2</sup> for concentrating the chorionic gonadotropin in the urine of pregnant women has proved highly efficient when *X. laevis* is used as a test animal for pregnancy. Since it was our intention to run a duplicate test on an African frog with each American frog used (in order to evaluate the reliability of the American frog as a test animal), use of the Scott concentration method provided an inoculum suitable for both animals, thus eliminating additional urine preparation. The urine was concentrated as follows:

One hundred cubic centimeters of a first morning specimen of urine are placed in a 100 cc graduated cylinder and acidified to a pH of 4.0 using 20 per cent HCl with 0.04 per cent bromocresol green as an indicator. When the urine specimen presented for testing is less than

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100 c.c., distilled water is added in the amount necessary for the recommended volume. Urine specimens of less than 50 c.c. are rejected as unsuitable, since our experience with several hundred tests on *X. laevis* has shown that such small quantities of urine do not usually contain the necessary titer of hormone to produce a positive reaction, especially in very early pregnancy. Also, urines with a specific gravity of less than 1.015 are usually unsuitable for testing, giving rise to a large percentage of false negatives unless a full 100 c.c. of urine are available. In the latter case a specific gravity of 1.010 is satisfactory. To the acidified urine, 5 c.c. of a 20 per cent suspension (in water) of acid washed kaolin are added, followed by thorough mixing (inversion of the cylinder twelve times is sufficient). Acid washed kaolin may be prepared,\* or it may be bought already prepared†. We have been using the Baker preparation with excellent results.

The cylinder is set aside until the kaolin has settled out to a volume of 15 to 20 c.c., at which time the supernatant urine is siphoned off and discarded. The sedimented kaolin is transferred to a test tube and packed by centrifuging at moderate speed for about five minutes. The resultant supernatant is discarded. Five cubic centimeters of 0.1N NaOH are added to the packed kaolin and the kaolin is redistributed through the alkali by vigorous stirring with a glass rod. When thoroughly mixed the kaolin alkali suspension is spun in the centrifuge at high speed for about five minutes or until the supernatant is clear. The clear supernatant is poured over into a test tube and made pink by the addition of 1 drop of 0.5 per cent alcoholic phenolphthalein solution. Twenty per cent HCl is added drop by drop until the pink color is just discharged. The solution is ready for injection. The foregoing procedure should consume no more than thirty to forty five minutes.

The use of the concentrated urine as a test inoculum for *R. pipiens* has given excellent results. One cubic centimeter of concentrated urine is injected into the dorsal lymph sac with no ill effects upon the animal observed. Since the 1 c.c. of concentrated urine represents a volume of 20 c.c. of whole urine its use is most advantageous in low hormone titer early pregnancy urine specimens. However concentration of the urine may lead to false positive reactions in menopausal urine specimens with high follicular stimulating hormone titers. Where menopause is suspected urine giving a positive reaction should be checked using either a rabbit or an African frog or by injecting two 2½ c.c. doses of whole urine into *P. pipiens* at one hour intervals.

#### TECHNIQUE FOR *R. PIPIENS* TEST

One hundred cubic centimeters of a first morning urine specimen are prepared by the Scott method and 1 c.c. of the final product is injected into the dorsal lymph sac of the frog (see Fig. 1). The animal is then placed in a clean dry glass receptacle (a 1000 c.c. beaker is ideal) covered with a weighted wire mesh top to prevent escape of the animal. At the end of one half hour, the frog is grasped firmly in the left hand, and the right leg of the animal is imprisoned between the operator's index and second fingers. The animal's left leg is taken between thumb and index fingers of the operator's right hand, and the leg is drawn up close to the frog's side. The frog's cloaca is then brought in contact with a clean glass microscope slide. The slide is immediately transferred to the microscope stage and the drop of urine examined under either the low or high dry objective. The sperm cells are easily recognized with either magnification. In a positive test, the sperm cells are shed in large numbers and can be seen actively thrashing about, or quiescent with their long whiplike tails waving slowly back and forth. In a negative test there are, of course, no sperm cells.

\*As described in *Practical Physiological Chemistry* Hawk & Bergelin ed. 11 page 63 footnote.

†From J. T. Baker Chemical Company Phillipsburg N. J. as Kaolin Washed Powder (American Standard).

The urine is quite clear, except for occasional red blood cells and one or more of a variety of intestinal parasites harbored by the frogs. These latter in no manner resemble the sperm cells and should not be mistaken for them. If at the end of the first half hour the urine shows no sperm cells, the procedure is repeated at half-hourly intervals until three hours have elapsed from the moment of injection. Absence of sperm cells within the three-hour period constitutes a negative test.

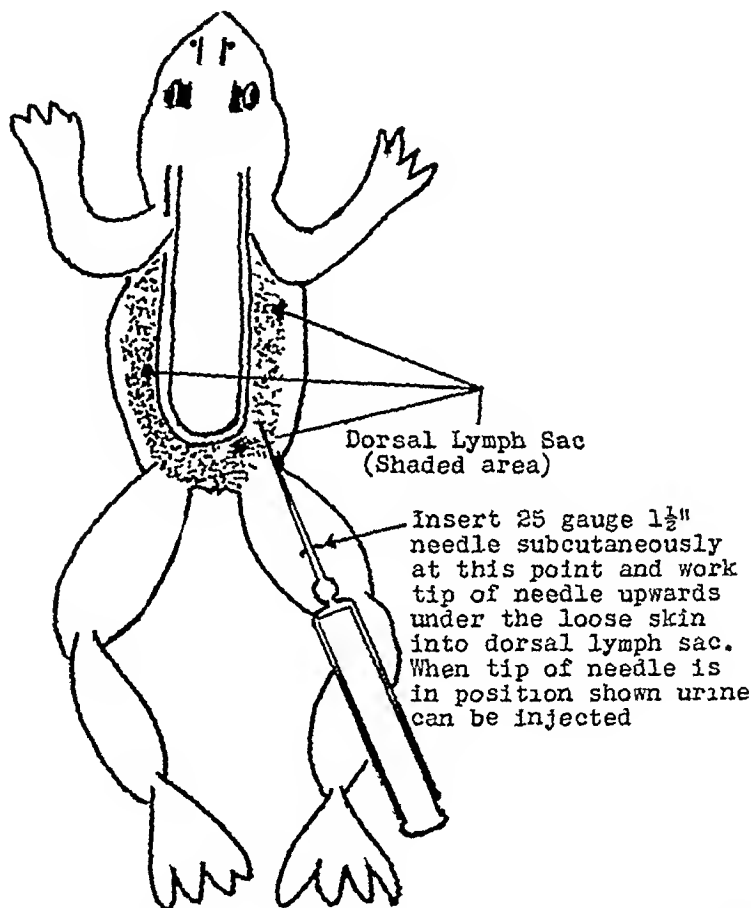


Fig 1—Diagram illustrating site and method of injection for *R. pipiens* pregnancy test

#### ACCURACY OF *R. PIPPIENS* AS COMPARED WITH *X. LAEVIS*

Unselected urine specimens brought to the laboratory were concentrated. A male *R. pipiens* and a female *X. laevis* were inoculated with the urine concentrate. Results were observed and compared.

In a series of 200 urine specimens from women suspected of being pregnant, two American frogs gave false negative reactions where *X. laevis* injected with the same urine specimens gave positive tests. Conversely, two *X. laevis* frogs gave false negative reactions with urine specimens that reacted positively on

the male *R. pipiens*. This constitutes a record of 99 per cent accuracy for both species of test animals since the combined results by the laboratory tests were all checked and substantiated by clinical findings. Of the 200 urine specimens tested, 130 were from women thought to be thirty to thirty five days pregnant (ten to fifteen days after first missed menstrual period) thus proving the ability of the *R. pipiens* test to detect conception as early as any of the tests now in routine use.

#### DISCUSSION

In our opinion, the pregnancy test using *R. pipiens* is far superior to any of the routine tests now in use. It is an exceptionally reliable test. In our analysis of 200 urine specimens only two false negative results were encountered. These two failings occurred early in our investigation when we were trying to reuse animals which had already given one positive test, after a five day rest period as recommended by Wiltheiser and Miller<sup>3</sup>. It was found inadvisable to reuse the animals for the following reasons: (1) A positive reaction appears to exhaust the frog's supply of sperm cells. Resting the frogs long enough to regenerate new sperm cells involves a time interval within which manition and wasting occur. This is due as Robbins and Parker<sup>1</sup> point out, to the impracticability of providing the proper live insect diet for the animals under laboratory conditions. (2) The animal is so inexpensive and its supply so readily available that it is false economy to salvage the frogs for reuse, thereby risking unreliable results. In cases where urine specimens have proved negative for pregnancy, we have reused the animals involved with consistently reliable results, but it is recommended that such animals be returned to their storage tank in the refrigerator and not kept at room temperature. After instituting the policy of not reusing previously positive animals 150 urine specimens were analyzed for pregnancy using *R. pipiens* as the test animal without any further false negative (or false positive) reactions.

The care necessary for the use of *R. pipiens* is simplicity itself. A small fish aquarium (14 in. by 10 in. by 8 in.) will easily house up to twenty frogs. Such a tank conveniently fits into the lower compartment of even a small laboratory refrigerator, where the animals are stored at about 10° C. in one half inch of water until ready to use. While in the refrigerator, the animals are in a state of hibernation and will survive for weeks on end without feeding. Aside from keeping the water level constant in the tank, there is no other care necessary for *R. pipiens*. One hour prior to use in a test, a frog is removed from the tank and placed in a suitable wire mesh covered receptacle at room temperature. This short thawing period is sufficient to return the animal to metabolic activity necessary for the test. In practice, we remove the frog for thawing as soon as the urine for testing reaches the laboratory. By the time the urine is concentrated and ready for injection, the frog is ready, too. Storage of *R. pipiens* at room temperature for more than a day or two is not recommended. At the beginning of our studies we stored all our frogs at room temperature. We found that wasting and manition were quite rapid, especially during the warmer summer months, but most disconcerting was the high spontaneous mortality rate of the unused frogs, at times as high as 50 per cent within two or three days.

after receiving the animals from our supplier. An occasional spontaneous death occurs among the refrigerated animals, but it is an infrequent occurrence.

The rapidity with which the results of the test develop is, in our opinion, the most remarkable feature of this new technique. Using 1 cc of the concentrated urine as our inoculum, we have observed positive reactions consistently within one-half to three-quarters of an hour after injection of the animal. Even allowing up to one hour for concentration of the urine, results of the test can be obtained in less than two hours from the time the specimen reaches the laboratory. This compares with a minimum of forty-eight hours for the standard Friedman test and up to eighteen hours for the *X laevis* test.

The advantages of using whole, unmodified urine in any pregnancy test are obvious. Although we attempted such a procedure, as described by the originators of the test, the results (mentioned elsewhere in this report) in our hands were discouraging. Robbins and Parker<sup>1</sup> describe the same difficulty with what they call "toxic urines," but apparently the rate of mortality from injection among their frogs was not as great as that which we encountered. They further decry the lack of adequate methods for detoxifying the urine specimens. It is our opinion that the large volume of the prescribed inoculum (5 cc) for such a small animal is responsible for the high frog mortality and not the presence of toxic substances in the urine. By use of the Scott method,<sup>2</sup> a small volume inoculum (1 cc) representing the hormone content of 20 cc unmodified urine is provided and at the same time substances toxic for the frog are removed. The latter is accomplished by the ability of acid-washed kaolin at pH 4.0 to selectively adsorb the protein materials from the urine, leaving behind the toxic substances in the supernatant which is discarded. This phenomenon has demonstrated itself to our satisfaction inasmuch as we have not had a single technical fatality among our frogs since substituting the concentrated specimen for whole unmodified urine. Once the necessary reagents for Scott's method<sup>2</sup> are at hand, the actual preparation of the urine for injection is neither technically difficult nor excessively time consuming, and the fact that each test goes to its desired conclusion without mishap, requiring the use of only a single animal, more than compensates for the necessary preparation of the urine.

The originators of the *R. pipiens* pregnancy test emphasize the fact that as yet they have not determined whether a seasonal variation in the reactivity of the frog might be encountered, especially during the breeding season. From their report, it is to be presumed that their investigations ran through the fall and winter of the year. Our experiments began in the early spring and continued throughout the summer months until the end of September. Apparently this would complete the cycle of one full year and would include the breeding season of the American frog. During the seven months from the beginning of March until the end of September, we encountered no variation in the reactivity of the animal to the gonadotropic hormones involved in the urine pregnancy test. These results, coupled with those of Robbins and Parker,<sup>1</sup> and Wiltberger and Miller,<sup>3</sup> would seem to indicate that *R. pipiens* can be used throughout the year as a reliable test animal for urine pregnancy testing.

## SUMMARY

The use of whole unmodified urine as a test medium for *R. pipiens* is not entirely satisfactory, resulting in the death (in our hands) of the animal when given the necessary 5 cc dose.

Concentration of the urine by Scott's method yields an ideal medium for *R. pipiens*. The medium is nontoxic with 100 per cent survival of the test animal throughout the testing period. Moreover, the accuracy of the test is enhanced by providing a greater concentration of hormone, a particularly valuable consideration in very early pregnancy.

Scott's method for concentrating the urine is described in detail as well as the actual technique in carrying out the test on *R. pipiens*.

Reuse of animals which have given a positive test may lead to false negative results in the second test. For this reason reuse of such animals is not recommended. Animals which upon previous testing yielded negative results may be reused safely after a twenty-four hour rest period.

An attempt to evaluate the reliability of *R. pipiens* as a test animal has been made by injecting a standard test animal (*X. laevis*) and a *R. pipiens* male frog simultaneously with the same urine specimen and then comparing the resultant reactions. Both species of animals produced a record of 99 per cent accuracy proved clinically.

The seasons of the year covered in this report (early spring to early autumn) in conjunction with the reports of other investigators working through the fall and winter would indicate that there is no seasonal or breeding period variation in the animal's response to gonadotropic hormone.

With modifications in the original technique (described in the body of this report), the pregnancy test using male *R. pipiens* frogs has proved itself in our hands, to be exceptionally reliable so much so that it has replaced *X. laevis* and rabbits in our laboratory. In addition, the shorter reaction period, the exceptionally low cost of the animal, the ready availability of supply, and the minimum of laboratory care and housing of *R. pipiens* make it in our opinion the animal of choice in urine pregnancy testing.

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# AN IMPROVED TECHNIQUE FOR THE TRANSMISSION OF THE LANSING TYPE VIRUS OF POLIOMYELITIS IN MOUSE EXPERIMENTS

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MANY attempts were made to shorten the incubation period of poliomyelitis infection in animals<sup>1 2 3</sup> Most of them were directed to the production of a *locus minoris resistentiae* in the central nervous system by traumatizing the brain tissue Another trend is to increase the amount of material injected by the intracerebral route This amount is, however, limited by topographic and special conditions Attempts to increase the usual dose administered in one injection from 0.01 ml to 0.03 ml have not been successful as yet According to our own experience, approximately 0.01 ml of the injected material is lost by back-seepage if 0.03 ml is injected Thus the problem is twofold first, there is a necessity to produce brain trauma by such means as will not kill the animal or weaken the virus, second, the amount of injected material has to be increased by administration in a different manner from the accepted and routine injection into one side of the brain

In order to achieve this end, it was decided to inject the infective material into both sides of the mouse cranium Different sites were selected as points at which to pierce the skull Preliminary experiments have shown that the largest number of mice survive when the injections are given exactly at a point halfway between the outer corner of the eye and the center of the external acoustic meatus

When the Lansing type of virus of poliomyelitis is transferred from mouse to mouse, brain emulsions are used It was mandatory, therefore, to use brain emulsions from normal, noninfected mice to determine the mortality rate due to trauma when using the described route of injection

One hundred twenty-five mice were inoculated with 0.03 ml of a 10 per cent emulsion of brain from normal mice on both sides of the cranium Only three (i.e., 2.4 per cent) of the mice died as a result of the inoculation Thus this method of administration seemed to be satisfactory

Most workers prefer clear suspensions when propagating the virus of poliomyelitis by injecting brain material of one mouse into another This is achieved by grinding the brain with aluminum or other suitable material in buffered saline, filtering through gauze, and centrifuging off coarse particles<sup>4</sup> Finally, the supernatant is inoculated into the brain of another animal

In addition to attempting to increase the brain trauma, we also tried to change the preparation of the infective material by the following means

The brain was ground with unbuffered saline in a mortar without aluminum or other similar material The volume of the saline used for this was ten times

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larger than the volume of the brain. After thorough grinding the material was poured into a test tube and left to stand in the icebox for thirty to sixty minutes. Then 0.03 ml of the supernatant was injected into each side of the head of the mouse by piercing the skull at a point halfway between the outer corner of the eye and the center of the external acoustic meatus.

Young, Plymouth strain Swiss mice were used for the experiments. The Lansing type of virus was received by the courtesy of Dr. S. O. Levinson, from the Michael Reese Research Foundation. Table I shows the results of experiments in which the classic technique and our modifications were compared. It is well evident from the tabulations that the technique described here gives superior results.

TABLE I

MATERIAL INOCULATED	NUMBER OF MICE	ID	INCUBATION PERIOD	AVERAGE TIME TO PRODUCE 50% MORTALITY	NUMBER OF MICE DEAD FROM TRAUMA
Lansing virus, crude suspension, bilateral inoculation	100	1 $\times 10^{-6}$	5 $\pm$ 3	4 $\pm$ 1	2
Lansing virus crude suspension, unilateral inoculation	40	1 $\times 10^{-5}$	10 $\pm$ 6	6 $\pm$ 2	1
Lansing virus in buffered saline pH 4.0, unilateral inoculation	40	1 $\times 10^{-4}$	12 $\pm$ 6	8 $\pm$ 2	1
Mouse brain, without virus bilateral inoculation	125	0	0	0	3

Number of days between inoculation and onset of paralysis

It has to be added that in spite of the larger traumatization due to the bilateral inoculation, the mortality of mice due to mechanical factors was very low.

While the results achieved by the use of this technique were very satisfactory, it has to be considered that the clinical picture of traumatism of the brain, i.e. contralateral paralysis developing early after the injection, cannot be evaluated in animals after material has been introduced into both sides of the cranium. This may cause difficulties in the evaluation of the results.

#### SUMMARY

An improved technique was described for the propagation of the virus of poliomyelitis from mice to mice. This method consists of injecting less purified suspensions in unbuffered saline bilaterally into the brain.

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# A METHOD FOR THE DETECTION OF LACTOSE IN URINE

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IT IS common experience in chemical laboratories that the published methods for the detection of lactose in urine are unsatisfactory. Fermentation tests are preferable to others but, unless carefully performed and interpreted, may lead to false conclusions. The chemical methods, which should serve at least to confirm the results of fermentation studies, are found to be wanting in both sensitivity and specificity. Among the many chemical procedures which have been described is the methylamine test<sup>1</sup>. Urine suspected of containing lactose is boiled with a small amount of methylamine and then made strongly alkaline with NaOH, a red color indicates the presence of lactose. As described, the method is not sensitive and results cannot be consistently reproduced.

A study of the factors affecting the methylamine reaction has indicated that, under the proper conditions, this test can be made very sensitive, and the results are so reproducible that, with certain refinements of procedure, it may be adapted to the quantitative determination of lactose. By the methylamine reaction we are able to detect quantities of lactose which are not detectable by the usual Benedict's test for reducing sugars.

## PROCEDURE

To 5 ml. of urine add 1 ml. of a 0.2 per cent aqueous solution of methylamine hydrochloride, and 0.2 ml. of 10 per cent NaOH. Mix by inversion or gentle swirling. Cover the tubes with glass bulbs or marbles and place in a water bath at 56° C. for thirty minutes. Remove from the bath and allow to stand at room temperature.

If the urine contains much lactose a red color will be apparent before the heating period is completed. The color increases as the tubes are allowed to stand at room temperature, reaching a maximum in about an hour. Hence, if the concentration of reducing sugar is low it is best to observe the tubes after they have been allowed to stand for some time.

## DISCUSSION

*Sensitivity*—An intense red color is observed after fifteen to twenty minutes at 56° if the urine contains 0.5 per cent lactose. This is a concentration which gives a Benedict's reaction usually interpreted as one plus. A 0.05 per cent solution of lactose in urine, which is negative with Benedict's reagent, gives a slight but definite positive reaction after standing for thirty minutes at room temperature. In order to detect traces of lactose it is best to run a control, containing water in place of methylamine, in order to avoid confusion caused by the color of the urine.

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*Other Sugars*—The only sugars which give a red color in the methylamine reaction are the reducing disaccharides. The only members of this group which have been found in urine are lactose and maltose. Maltose, which has been reported as occurring in a few instances, seldom has been identified definitely. Glucose, fructose, xylose, galactose and sucrose in large amounts give a yellow color which is readily distinguished from the lactose reaction.

*Temperature and Alkalinity*—Although a more intense color is produced at higher temperatures the interpretation of the results is made difficult because of an interfering color caused by caramelization of the sugar. A temperature of 56° maintained for thirty minutes gives an intense color with lactose and causes little caramelization as shown by the slight color of the blank. This temperature was chosen because it is close to the optimum and baths at this temperature, used for inactivation of serum, are generally available in clinical laboratories.

The optimum amount of NaOH was found to be 0.2 to 0.4 ml. of a 10 per cent solution. Since, however, the larger amount causes an increased blank color, it is advisable to use only 0.2 milliliter. If the amount of 10 per cent NaOH added is either larger or smaller than 0.2 to 0.4 ml., the intensity of color given by lactose is decreased.

*Aeration*—It was noted in the original description of the methylamine reaction<sup>1</sup> that the color is decreased if the tubes are violently shaken. This result has been confirmed and it is therefore advisable to avoid aeration. For this purpose the contents of the tubes are mixed gently and, during the heating period, are kept covered.

*Effect of Ammonia*—The concentration of ammonia has a marked effect on the intensity of color produced by a given amount of lactose (Table I). In the table the amount of methylamine added is smaller than that recommended when using the reaction to detect lactose in urine. The concentrations of methylamine and lactose were adjusted to give intensities which could be read on the Klett-Summerson colorimeter, using a No. 54 filter.

TABLE I. 5 ML. 0.3 PER CENT LACTOSE, 0.5 MG. OF METHYLAMINE HYDROCHLORIDE, 0.2 ML. 10 PER CENT NaOH, HEATED AT 56° FOR 30 MIN., READ AFTER STANDING 1 HR.

Mg. NH <sub>3</sub> -N added	0	0.1	0.4	0.5	1.0	5.0	10.0	15.0	20.0
Readings	8	86	91	109	121	246	332	0	0

It is evident that increasing amounts of ammonia up to 5 to 10 mg. enhance the color, but if addition of ammonia is continued, color development is completely inhibited. Ammonia, in the absence of lactose, gives no red color, but appears to increase the yellow color ascribed to caramelization; this effect again increases until 10 mg. of NH<sub>3</sub>-N have been added and then falling off to zero.

It is unlikely that the presence of ammonia in fresh urine would cause inhibition of color development from lactose, since this would require greater than 2 mg. ammonia-N per milliliter of urine, an unlikely concentration. It is possible, however, that if urine is allowed to stand until bacterial decomposition

of urea has progressed markedly, that a concentration of ammonia great enough to produce inhibition might be reached. In such a case the ammonia is readily removed with permutit.

*Quantitative Determination of Lactose*—Preliminary experiments have indicated the feasibility of applying the methylamine reaction to the quantitative determination of lactose. In this case the factors of temperature and alkalinity must be carefully controlled. In addition, the amount of ammonia present must

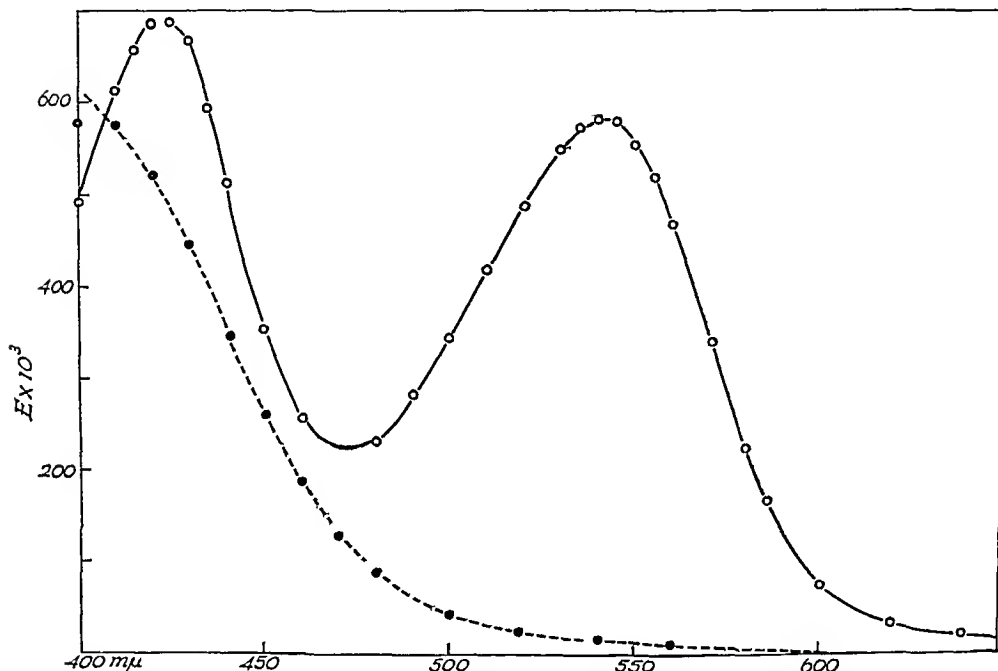


Fig 1—Absorption spectrum of color produced in the methylamine reaction. The broken line represents the spectrum of the blank. A Cenco-Sheard Spectrophotometer with a 1 cm cuvette was used.

be known and adequate compensation provided. The ammonia may be determined and enough added to the standards to equal the amount present in 5 ml of urine. A better procedure appears to be the removal of ammonia from the urine with permutit, and then the addition to blank, standards, and urine sample of 5 mg of ammonia-N, thus utilizing the ammonia to increase the sensitivity of the method.

If the method is to be used quantitatively it is necessary to compensate for the color of the urine by using a blank containing urine, but no methylamine. A determination of the absorption spectrum of the colored substance (Fig 1) shows two absorption maxima, at 425 and 540 millimicrons. Although there is greater absorption at 425  $m\mu$ , the blank also shows great absorption at this wave length, whereas at 540  $m\mu$  the blank exhibits scarcely any absorption. Readings are, therefore, made with a filter having its greatest transmittance at 540 millimicrons.

## SUMMARY

1 The methylamine reaction can be utilized, under the correct conditions for the detection of small amounts of lactose in urine

2 Preliminary experiments indicate that the method might be adapted to the quantitative determination of lactose

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## A VISCOSITY-EFFUSION METER FOR MEASURING THE CONCENTRATION OF ANESTHETIC GASES

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PUBLISHED methods for estimating the concentration of ether and other anesthetic gases in air, or air plus oxygen, are not suitable for routine use in the operating room. For determining ether both chemical and physical analytic procedures have been described. The former include the combustion method of Spenzer,<sup>1</sup> the method of Nicloux,<sup>2</sup> later modified by Shaffer and Ronzoni,<sup>3</sup> in which the ether vapor is absorbed in 50 per cent sulfuric acid and subsequently oxidized with dichromate, and the iodine pentoxide method of Haggard.<sup>4</sup> The time required to complete such chemical analyses renders the results of little immediate use to the anesthetist. Physical methods which measure the increase in gas density caused by the presence of ether have been reported by Boothby,<sup>5</sup> who used the Waller<sup>6</sup> gas balance, and by Harger and co-workers,<sup>7</sup> employing a modified Cady<sup>8</sup> gas balance. These vapor density methods yield immediate results, but the necessary apparatus is too cumbersome to be used except for research where trained technicians are available to operate the gas balance. Kruse<sup>9</sup> determined ether in air by means of the Guthrie analyzer,<sup>10</sup> using concentrated sulfuric acid to absorb the ether, and Seevers and associates<sup>11</sup> used the gas analysis apparatus of Burrell, Seibert, and Jones<sup>12</sup> to determine cyclopropane in air, employing fuming sulfuric acid as the absorbent for cyclopropane. These methods, which measure contraction in gas volume following absorption of the anesthetic gas, require a technician and take some time.

In an effort to develop a very simple, rapid, automatic analytic method for anesthetic gases we have experimented with a number of physical procedures. We first used twin flowmeters operated in series with a tube containing activated charcoal between them to absorb ether vapor, the apparatus being essentially that of Strache and co-workers<sup>13</sup> as modified by Sauter.<sup>14</sup> This gave fairly good results but required the constant use of the absorbent. Next, we tried methods depending upon the decrease in viscosity produced by ether, either by measuring the rate of flow through a capillary tube at constant pressure, or by determining the change in pressure between the capillary ends at constant flow. We then substituted for the capillary tube a fine orifice in a thin plate\* which would measure change in rate of effusion caused by alteration of gas density, in conformity to Graham's law.<sup>15</sup> The effusion molecular weight apparatus of Eyring<sup>16</sup> is a somewhat recent application of this principle. In our hands both viscosity

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\*We are indebted to Mr. Charles Ness of the Linde Air Products Company, Indianapolis, Ind., for constructing our first orifices.

and effusion methods gave quite satisfactory readings but it was found somewhat difficult to maintain constancy of an flow or pressure differential.

Finally, we found that these difficulties could be avoided by an arrangement which registers the combined changes in viscosity and density. Our apparatus is a modification of the device described in 1920 by Viehoff<sup>17</sup> for determining the concentration of carbon dioxide in flue gas. In Viehoff's apparatus the gas to be analyzed is drawn through a capillary tube and then through a fine orifice. Using a common suction, ordinary air is drawn through an identical tube and orifice, and a manometer connecting the two limbs by means of side arms placed between the capillary and orifice registers any pressure difference between the two limbs. Since carbon dioxide has a higher density than air its presence reduces the rate of effusion through the orifice in the flue gas limb, while the lower viscosity of carbon dioxide as compared with air causes a decrease of the resistance to the passage of gas through the corresponding capillary tube. These changes in effusion and viscosity are additive and result in an increase of gas pressure in the flue gas arm of the apparatus thus producing a rise in the manometer on the air side. According to Dennis and Nichols<sup>18</sup> the presence of moisture and other impurities interferes somewhat with the accuracy of the method when used for flue gas. The Viehoff viscosity effusion bridge has been employed by Jenkins<sup>19</sup> for determining the levels of both oxygen and carbon dioxide inside an oxygen therapy chamber.

Since ether also has a higher density and a lower viscosity than air or oxygen, the differences being much greater than for carbon dioxide, this procedure somewhat modified was found to be admirably suited for the determination of ether.

When using the Viehoff apparatus to analyze the rebreathed gas from an anesthetic machine, one cannot employ air as a reference gas since the oxygen content of the rebreathed gas is frequently far above 21 per cent, so that an absorbent must be constantly used for one limb. For this reason we have modified the apparatus by using only one of the twin limbs and connecting the manometer to the two ends of the capillary tube. The presence of ether causes a marked drop in the manometer level. To establish the base line the inlet is provided with a two way stopcock by means of which one can momentarily shunt the gas through a tube containing charcoal or other suitable absorbent. This base line changes rather slowly during a surgical operation so that an occasional adjustment will suffice. In order to maintain a constant flow for a given gas mixture, the suction employed distal to the orifice is kept above the critical point of about 40 cm of mercury (Saint Venant and Wantzel<sup>20</sup>). The apparatus is extremely simple and operates automatically, giving prompt continuous readings of the concentration of ether vapor.

The method was found to be satisfactory for the determination of nitrous oxide, cyclopropane, and ethylene in the presence of nitrogen or oxygen. Nitrous oxide and cyclopropane each has a higher viscosity but a lower density than oxygen or nitrogen while ethylene has about the same density as air but a much lower viscosity. With a ternary mixture of nitrogen, oxygen and one of these

gases the per cent of oxygen must also be determined, but this can be accomplished quickly with the Pauling-Beckman apparatus<sup>21</sup> Helium in an oxygen may be measured by our effusion-viscosity device The much lower density of helium causes a marked rise in the manometer column The apparatus will also indicate the approximate percentage of oxygen in nitrogen, but with the manometer used for ether the change in level is small The reason for this is that the density and viscosity of oxygen are both slightly higher than for nitrogen so that the two changes act oppositely Since the viscosity effect is greater than the density effect, the result is a small rise in manometer reading when oxygen is added to nitrogen

One also can use the apparatus to determine both nitrous oxide and ether and cyclopropane and ether when these pairs are present in the gas mixture, since one may selectively remove the ether, thus obtaining a second reading for the ether-free gas However, since the total manometer drop exceeds the sum of the drops due to ether and nitrous oxide or ether and cyclopropane, it is necessary to employ a table or nomogram constructed from observed manometer readings with various mixtures of ether-nitrous oxide or ether-cyclopropane in an oxygen Our data for this purpose are not yet complete

#### DESCRIPTION OF APPARATUS

The type of apparatus which we have used is shown in Fig 1 The example described has an air flow of about 100 c c per minute, and a manometer height of 52 cm, which gives a drop of about 1 cm for each per cent of ether For compactness it is mounted on a vertical board 24 in long and 5 in wide, having a horizontal base 8 in long located on the back and about 10 in from the lower end In this way the apparatus projects only about 16 in above the table on which it is clamped, a part of the manometer being below the table level A small shelf for supporting the manometer bottle is located at the bottom of the vertical board The various parts are as follows

*Capillary Tube*—This tube (*A*) is 50 cm long with a bore of 0.67 mm and with the intake end sealed to a glass H For conserving space the capillary is bent in the form of a U, with care taken to avoid constricting the bore while bending The outlet from the capillary (*A*) is connected to the orifice (*B*)

*Variable Resistance to Gas Flow (D)*—This arrangement, described by Hofsass,<sup>22</sup> is placed at the intake of the capillary tube The variable resistance is a piece of 27 gauge (0.4 mm diameter) nichrome or platinum wire extending a short distance into the lumen of tube (*A*) The position of the wire may be adjusted by means of a gastight metal screw attached to the top of the wire Since the only gas pressure at this point is that required to draw the gas through the absorption tubes, it is not difficult to secure a gastight joint for this adjusting screw One can use a small glass rod sealed to the wire, which slides inside a rubber sleeve

*Orifice*—The orifice (*B*) is placed between the exit of capillary (*A*) and the suction tube (*C*) This orifice has a diameter of 0.12 mm and is drilled in a sheet of platinum 0.01 in thick, using a special drill for this purpose\* The sheet of platinum is bolted between two opposing brass plates so that the orifice is between two brass tubes soldered into holes in the centers of the plates Gaskets of thin sheet tin are used to insure tightness A plug of cotton is placed in the line immediately ahead of the orifice so that dust particles will not collect on the orifice and change its diameter

\*This drill was obtained through the kindness of the National Jet Company Cumberland Md

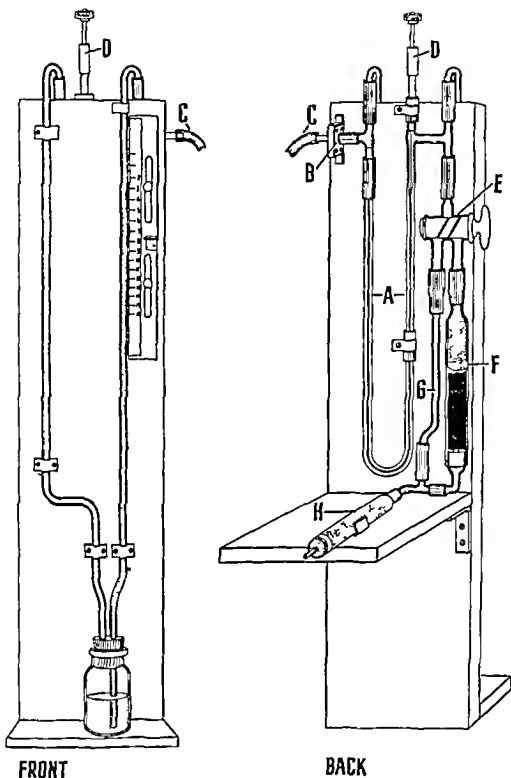


Fig 1—Diagram of viscosity effusion meter

**Manometer**—This is connected to side arms just beyond the two ends of the capillary tube. It is of the reservoir type the glass tubes having an inside diameter of about 3 millimeters. The reservoir is a 2 oz. wide-mouthed bottle with an inside diameter of about 3.6 cm and is fitted with a two-hole rubber stopper. In this way more than 99 per cent of the shift in manometer level occurs in the glass tube on the right. We use a free-flowing manometer oil having a specific gravity of 0.827 and containing a red dye.\*

**By-pass for Absorption**—A two-way stopcock (E) with a 2 mm bore is attached to the inlet end of the capillary (A) so that the gas to be tested may enter through the plain tube (G) or through the absorption tube (F) which contains a layer of the absorbent followed by calcium chloride. The reason for the calcium chloride in tube (F) is that charcoal and other absorbents frequently give off some moisture. Stopcock (E) is lubricated very lightly with a silicone grease, avoiding any of the lubricant in the lumen.

\*This manometer oil is sold by The Meriam Instrument Company, Cleveland 9, Ohio.

*Tube for Removing  $H_2O$  and  $CO$* —This tube (*H*) through which the gas first passes is filled with soda lime followed by calcium chloride. None of the anesthetic gases which we have tested is absorbed by calcium chloride or soda lime.

*Source of Suction*—One can use an ordinary good water aspirator with a trap in the line to prevent the backflow of water due to changes in water pressure. Since the apparatus as described has a flow of only about 100 c.c. per minute, we have used as a portable source of suction a strong 10 gal. steel drum which had been evacuated with a water pump. This drum is provided with a closed end mercury manometer so that one can make sure that the pressure inside it is always less than about 350 mm. of mercury. To avoid collecting ether vapor in this drum we use a large absorption tube containing charcoal which is placed in the suction line (*C*) between the drum and the orifice.

*Joints Between Parts*—All joints through which the gas passes before it escapes through the orifice should be as nearly glass to glass as possible since rubber rapidly absorbs ether and other anesthetic gases. Such absorption causes a lag in reaching the correct reading.

*Connection for Anesthetic Machine*—This is a metal tube about 3 in. long, machined to fit between the end of the hose from the mask and the metal hose connection of the machine. This tube has a metal side arm with an internal diameter slightly larger than that of a piece of capillary tubing with about 2 mm. bore which connects the inlet of tube (*H*) with the breathing tube of the anesthetic machine. This glass connecting tube is pushed inside the side arm of the metal sleeve so that its end is flush with the lumen of the breathing tube, and the connection between the glass and metal tubes is made gastight by means of a rubber sleeve.

#### PROCEDURE

Unscrew adjusting rod (*D*) so that the lower end of the wire is in the enlarged portion of the capillary tube (*A*). Connect the vacuum source to (*C*) with air entering at (*H*). The exact degree of partial vacuum is immaterial, so long as it is below 40 cm. of mercury. The manometer should now register 45 to 48 cm. above the reservoir level. Lower the wire of (*D*) until the manometer reads 52 centimeters. This is the *reference point* for all measurements with this particular apparatus. Mark this point on a sheet of cardboard mounted next to the manometer tube. Connect the inlet with the gas mixture to be analyzed, opening the stopcock of the gas container before connecting it to the apparatus. Turn stopcock (*E*) to shunt the gas through the absorbent in (*F*). After waiting about thirty seconds to allow the entering gas to flush out all gas previously in this limb of the apparatus, read the manometer level. If it is not at the reference point of 52 cm., adjust the variable resistance (*D*) until the level is exactly at this point. Now turn stopcock (*E*) so that the gas enters through tube (*G*), making sure that the stopcock lumen is fully open. When the manometer reading is constant, which usually occurs in about fifteen seconds, record the fall, or rise, from the reference point, measured in centimeters. Repeat the procedure with various other concentrations of the anesthetic gas and use the data to plot concentration against manometer deviation from the reference point. The resulting curve will approach a straight line. This curve may be used to construct a scale for the instrument, the other scale being shown in Fig. 1.

The absorbent for ether alone should be 12 to 24 mesh activated charcoal, which will absorb about one third of its weight of ether before allowing any to pass. With cyclopropane, tube (*F*) is replaced with an absorption tube containing pumice impregnated with concentrated sulfuric acid. For mixtures of ether and cyclopropane the apparatus inlet is provided with a third limb containing an activated fuller's earth absorbent, Florisil,\* followed by calcium chloride, and tube (*F*) contains pumice impregnated with sulfuric acid. When the gas is shunted through tube (*F*), this absorbs both ether and cyclopropane. When shunted through the third tube (not shown in Fig. 1) the Florisil absorbs ether but, except for the first few seconds, no cyclopropane.

\*The Florisil was kindly furnished by the Floridin Company, Warren, Pa.



For nitrous oxide and ethylene the manometer depression is read with the reference point set for air or oxygen. This is because we have found no satisfactory absorbent for either of these gases. Furthermore their concentrations during anesthesia are usually too high for them to be removed for any length of time.

#### PREPARATION AND STORAGE OF SPECIFIC GAS MIXTURES

Since ether and other anesthetic gases readily enter and pass through rubber and other plastics and dissolve in water and most other fluids except mercury, it is necessary to mix and store these gases in materials which are impervious to them. Our gas mixtures were prepared and stored either in flexible aluminum bags devised by us or over mercury in a glass container. These two procedures will be briefly described.

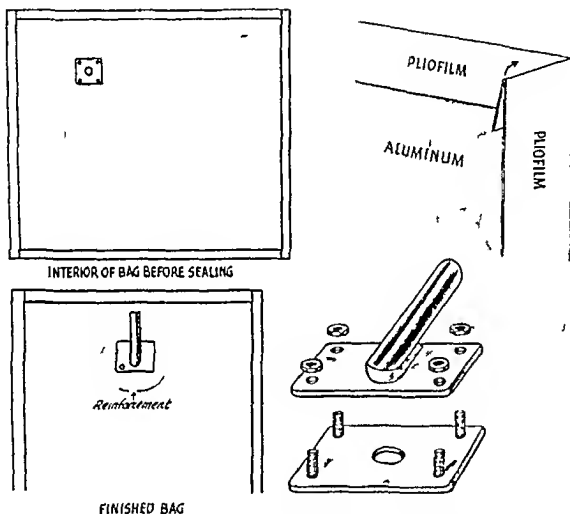


Fig. —Parts and assembly of flexible aluminum gas bags

*Flexible Aluminum Gas Bags*—The parts and construction of these bags are shown in Fig. 2. The bag material is sheet aluminum 0.001 to 0.0015 in. thick, covered on one side with a heat sealing plastic (Phofilm or Metalum). The material was purchased in a 100 ft. roll 18 to 36 in. wide\*. This sheet aluminum is manufactured for hermetically sealing coffee, drugs or articles which may be injured by exposure to moist air. As commonly used, two identical rectangles are cut from the sheet and the substance to be preserved is placed between them with the plastic surfaces facing each other. Heat from a laundry iron is then applied around the periphery of the sheets which causes them to seal together where heated, forming a gastight joint. Since we found that ether was somewhat absorbed by the plastic coating, we employed an arrangement involving a minimum of exposure of the plastic on the interior of the bag. A rectangle a little over twice the size of the finished bag is cut

\* This sheet aluminum coated with a heat sealing plastic was purchased from The Debeckum Company, Cleveland, Ohio.

from a sheet. This is laid on the table with the plastic side down. Along each edge of the sheet a strip about  $\frac{3}{4}$  in wide is then folded over and pressed down as shown in the sketch. The sheet is then doubled together to form the bag, making sure that the exposed plastic coated strips on the upper half are superimposed on those of the lower half. Before the sealing operation, a reinforcement for the inlet is sealed to the outside of one side of the bag, holes of proper size are cut in this reinforced portion, and the inlet is bolted in place, using thin sheet tin gaskets to insure tightness. The two halves are then sealed together.

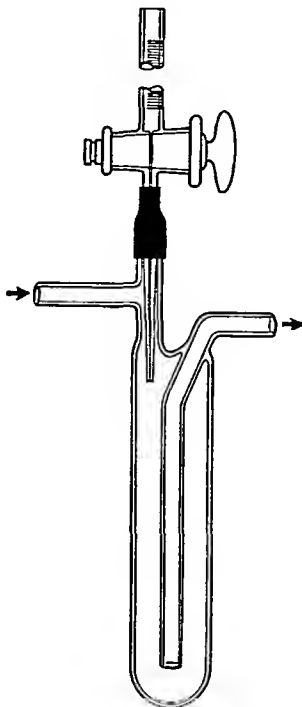


Fig 3—Ether vaporizer

with a hot laundry iron, using a sheet of paper between the iron and the plastic surface, after which a binder about 1 in wide is sealed to the four edges of the bag. If properly made these bags are absolutely gastight. After continued use they develop small pinhole leaks and should be discarded. However, a bag having a few very fine leaks may still be used provided the gas inside it is kept under slight pressure by placing a newspaper or rather large towel on top of the bag. As valves for these bags we use an unlubricated, well ground, Pyrex stopcock. The glass tube of the stopcock should fit fairly snugly inside the copper inlet tube and should be pushed two or three inches into this tube using a rubber sleeve to make a tight joint between the two. In this way very little gas inside the bag comes in contact with the rubber sleeve. When stored in these bags, mixtures of air, oxygen, or nitrogen with ether or the other anesthetic gases retained a constant composition for several hours.

*Filling the Bags With Specific Gas Mixtures* With ether, the liquid (absolute) is run from a microburette into a closed glass vaporizing tube about  $4\frac{1}{2}$  in by 1 in (Fig 3) with its lower half placed in water heated to around  $60^\circ$ . A short vertical inlet tube with a side arm is sealed to the top of the vaporizing tube. The microburette has a long, tapering tip which extends through this vertical tube, the joint between the burette and vertical tube being closed with a rubber sleeve. A second tube, which serves as the outlet, extends almost to the bottom of the vaporizing tube. Air, oxygen, or nitrogen to be mixed with the ether

enters the side arm of the inlet tube and the mixture with the ether vapor then emerge through the outlet tube which connects to the gas bag with all joints as nearly glass to glass as possible. The introduction of the liquid ether should be finished shortly before the last of the other gas is run through the vaporizing chamber in order to transfer all of the ether vapor to the bag. A calibrated flowmeter measures the rate of flow of the gas entering the vaporizing tube. One cubic centimeter of liquid ether when vaporized will occupy 232 cc at 21 and 760 millimeters. From this figure corrected for existing conditions of temperature and barometric pressure one can calculate the volume of liquid ether required to prepare the desired concentration of the vapor after it is mixed with a given volume of the other gas. In calculating the resulting per cent of ether vapor in the mixture one must correct for the increase in volume due to the addition of ether vapor. After being filled with the gas mixture, the bag should be gently kneaded to mix the contents. To further insure complete mixing the gas should be allowed to remain in the bag about ten minutes before it is used.

With compressed anesthetic gases the tank valve or a suitable reducing valve is connected to a flowmeter calibrated for this particular gas. The stream of gas from this flowmeter enters a Y tube where it meets a stream of air, oxygen or nitrogen the speed of which is also controlled by means of a second flowmeter. When the rates of the two gas streams are properly regulated the outlet of the Y tube is connected with the gas bag. When using this procedure we commonly prepare from 1 to 10 liters of the gas mixture.

As an alternate method for measuring the required volume of a given gas, one may employ a dry gas meter constructed from a 100 cc hypodermic syringe by cutting off a suitable length of the inner end of the plunger and using this section of the plunger as a piston discarding the remainder of the plunger. The piston should be of such a length that it will close the hole in the syringe when the open end of the syringe serves as a stop when the inner end of the plunger exactly reaches the 100 cc mark. The open end of the piston is closed with a well fitting cork which is inserted so that its exposed end is just flush with the end of the piston. If the piston is left open it will take a longer time to flush out the apparatus when changing gases. The syringe is mounted in a horizontal position. The two ends of the syringe are connected by glass tubes to a special stopcock which is bored to reverse the direction of flow for each 90 degree turn of the handle. To avoid a significant increase in pressure within the syringe while turning the stopcock a dry 50 cc hypodermic syringe is mounted in a vertical position with its opening at the bottom and connected through a T tube to the line between the tank and the syringe gas meter. Any gas which collects in this vertical syringe at the end of an excursion of the piston in the 100 cc syringe will be added to the latter syringe in the next stroke of the piston. With a well made syringe the gas pressure required to operate this meter is no greater than that needed for a standard wet gas meter, and there is practically no gas leakage between piston and barrel. This apparatus equipped with an automatic reversing valve will be described in a separate paper. The graduations on the 100 cc syringe should of course be properly checked with a good gas burette.

*Glass Chamber for Preparing and Storing Gas Mixtures Over Mercury*—This apparatus is shown in Fig. 4. Our gas chamber has a volume of 1125 cc with the mercury level at (X). A dry, well fitting Pyrex stopcock with 4 mm bore (1) is sealed to the top of the chamber. Just beyond the stopcock the tube branches. The vertical limb (B) is for inserting the tip of a 2 cc burette (C) through the bore of the stopcock (1). A rubber sleeve makes a gastight joint between tube (B) and the burette. Sealed to the horizontal limb is the female portion of a 12/30 standard taper Pyrex joint (D). The male portion of joint (D) is sealed to a glass T the long arm of the T being a capillary tube (E) with 2 mm bore which serves as delivery, or inlet for the chamber. The dry glass joint which is held together with rubber bands permits a vertical motion of tube (E). The horizontal limb of the T (F) is constricted and joined through a rubber sleeve to the outlet of a dry 100 cc glass syringe with part of the plunger cut away leaving the remainder as a free moving piston. This piston serves as a pressure equalizer so that gas leaving the chamber is always at atmospheric pressure. The pressure tubing connecting the chamber and the leveling bottle is fitted with a screw hose clamp.

*Ether Mixtures* The gas to be mixed with ether is stored in an aluminum bag and is kept under a slight positive pressure by placing a magazine or large towel on the bag. Open stopcock (A) and elevate the leveling bottle. Unscrew the hose clamp and allow the mercury to completely fill the chamber and ascend past the stopcock almost to the level of (D). Close stopcock (A) and the hose clamp. Connect the gas bag to (E) leaving the bag valve closed. By means of a 50 cc dry syringe withdraw gas through (B) until syringe (G) is empty. Now open the gas bag valve and withdraw about 50 cc

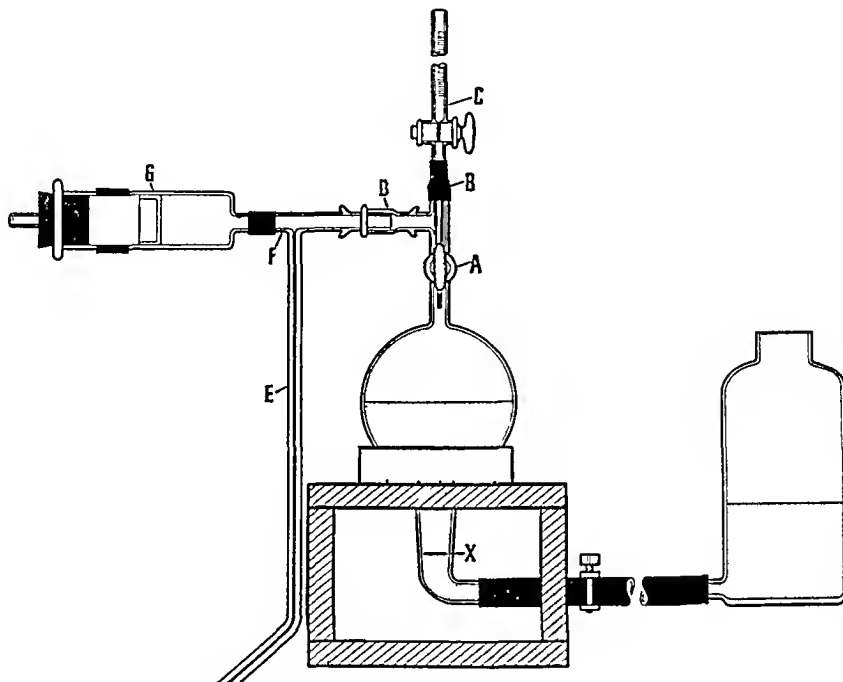


Fig. 4—Glass chamber and accessories for preparing and storing gas mixtures over mercury

of gas to flush out the tubes. Disconnect the 50 cc syringe and quickly close (B) with a glass rod. Completely open stopcock (A), lower the leveling bottle, and momentarily open the hose clamp to permit the mercury to descend about an inch, and then close the hose clamp. Remove the rod from (B) and quickly insert the burette (C) which contains anhydrous ether, making sure that the ether fills the tip. The burette stopcock is lightly lubricated with a silicone grease. The tip of the burette should project about 1 cm below stopcock (A). Slightly unscrew the hose clamp to permit a slow inflow of the gas during the introduction of the ether, and then run in the desired volume of liquid ether. Next, with the gas still entering slowly at (A) quickly remove the burette and close (B) with the glass rod. Close stopcock (A) and lower the mercury level to about the middle of the chamber so as to vaporize all of the ether. After about five minutes open stopcock (A), unscrew the hose clamp, slowly lower the mercury level exactly to (X) and then close the hose clamp. As soon as the gas in the chamber reaches atmospheric pressure, close stopcock (A). Allow the mixture to stand fifteen minutes to insure complete mixing. From the chamber volume (1,125 cc) and the volume of liquid ether introduced, calculate the per cent of ether vapor in the mixture. Checks of such mixtures with a modified Cady gas balance and other methods showed excellent agreement between calculated and observed results. Once made, such a mixture will retain a constant composition for days.

*Mixtures of Other Anesthetic Gases* As with ether, the diluting gas is stored in an aluminum bottle. Accurately measure the necessary volume of the anesthetic gas with the syringe gas meter previously described or by means of a mercury filled gas burette and then introduce it through tube (B). Run in the diluting gas through tube (E) as with ether.

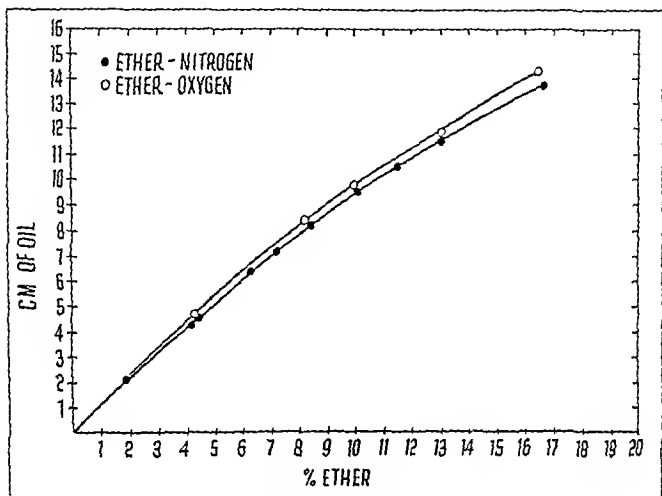


Fig 5—Manometer drop for various concentration of ether in nitrogen and in oxygen (5. cm base line)

Before connecting delivery tube (h) to the viscosity effusion meter close tube (B) with a glass rod and withdraw gas from (E) until syringe (G) is empty. Then open stop cock (A) and with the leveling bottle somewhat elevated slightly open the hose clamp to permit a slow escape of gas from the chamber. Next turn on the viscosity effusion meter with air passing through it and then connect the inlet tube of the meter with tube (E). Regulate the flow of mercury into the chamber so that the piston of syringe (G) is about at the mid point. This piston greatly facilitates the transfer of gas from the chamber to the meter.

**Caution regarding gas mixtures!!!** Ether cyclopropane and ethylene when mixed with air or oxygen are highly inflammable and in contact with a hot spark they will explode with great violence. Avoid flames or sparks in the vicinity of such mixtures!!!

#### VISCOSITY EFFUSION METER READINGS WITH KNOWN CONCENTRATIONS OF ANESTHETIC GASES

The results with ether in the presence of nitrogen or oxygen are shown in Fig 5. The mixtures were prepared and then composition checked, as described in the preceding section. It will be noted that the readings for ether in oxygen are about 3 per cent higher than those for ether in nitrogen, with both curves approaching straight lines. Since the oxygen content of re-breathed gas during anesthesia with ether is usually at least 50 per cent, the scale for ether was constructed from a curve midway between the ether nitrogen and the ether oxygen curves, which was shown to be the curve for ether in 1 1

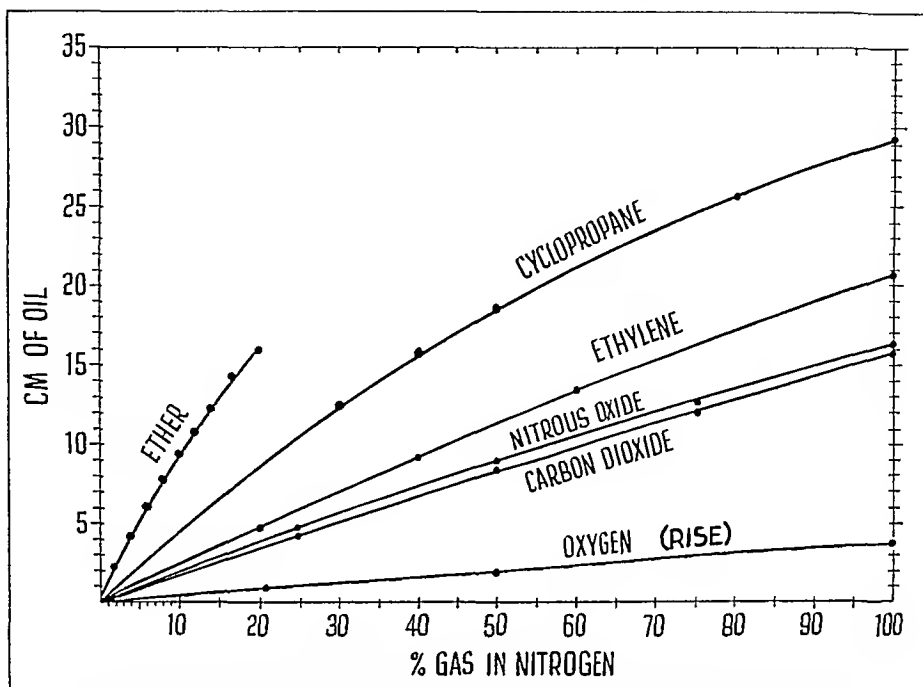


Fig 6 —Manometer shifts for certain gases in nitrogen (52 cm base line)

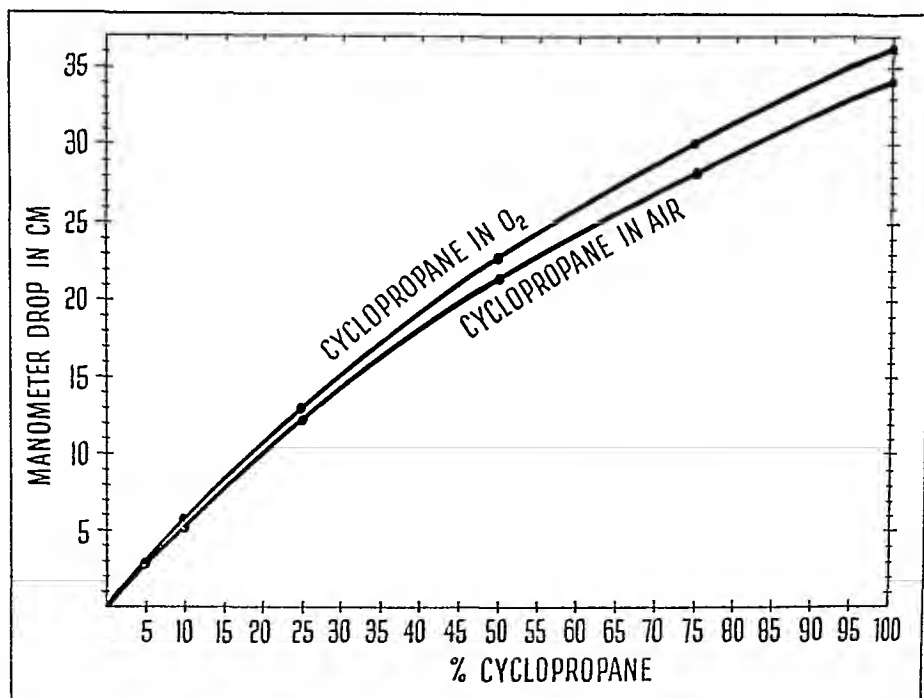


Fig 7 —Manometer drop produced by cyclopropane in air and in oxygen (62 cm base line)

N + O In this way the readings are within 99 and about 101 per cent of the correct figure

Fig 6 gives the results for binary mixtures of nitrogen with ether, cyclopropane, ethylene, nitrous oxide, carbon dioxide, and oxygen. In the case of oxygen the results represent a rise in manometer reading, the others being a fall. In all cases the reference point was set at 52 cm with nitrogen only. If

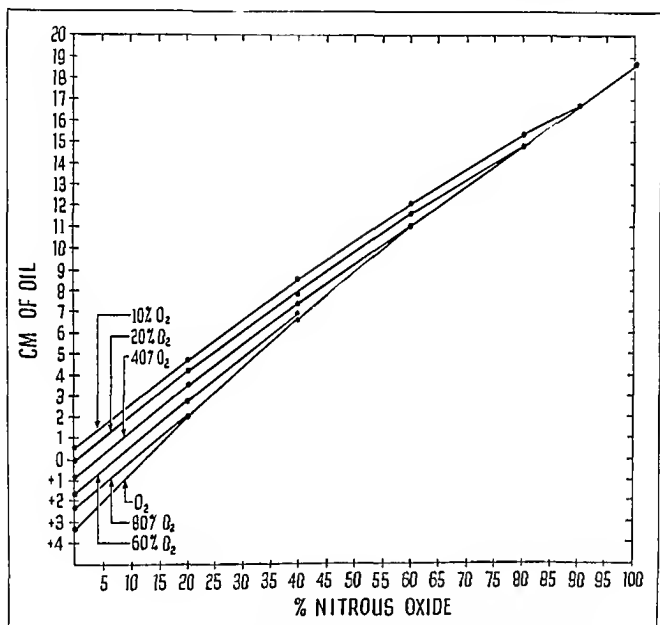


Fig 8—Manometer drop for ternary mixtures of nitrous oxide, nitrogen and oxygen (62 cm base line with reference point set for air. Oxygen percentages are for cyclopropane free gas.)

the manometer depression for ether in nitrogen is expressed as 1.0, the relative depressions produced by the same concentrations of the other gases are, respectively: cyclopropane, 0.51; ethylene, 0.29; nitrous oxide, 0.22; and carbon dioxide, 0.20, with a rise for oxygen of 0.043.

Various mixtures of cyclopropane and cyclopropane oxygen were run with a second viscosity diffusion meter having a reference point of 62 centimeters. The results (Fig 7) show that the readings with oxygen are all about 8 per cent higher than with air. In determining cyclopropane during anesthesia one should also determine the per cent of oxygen and use a curve of the type of Fig

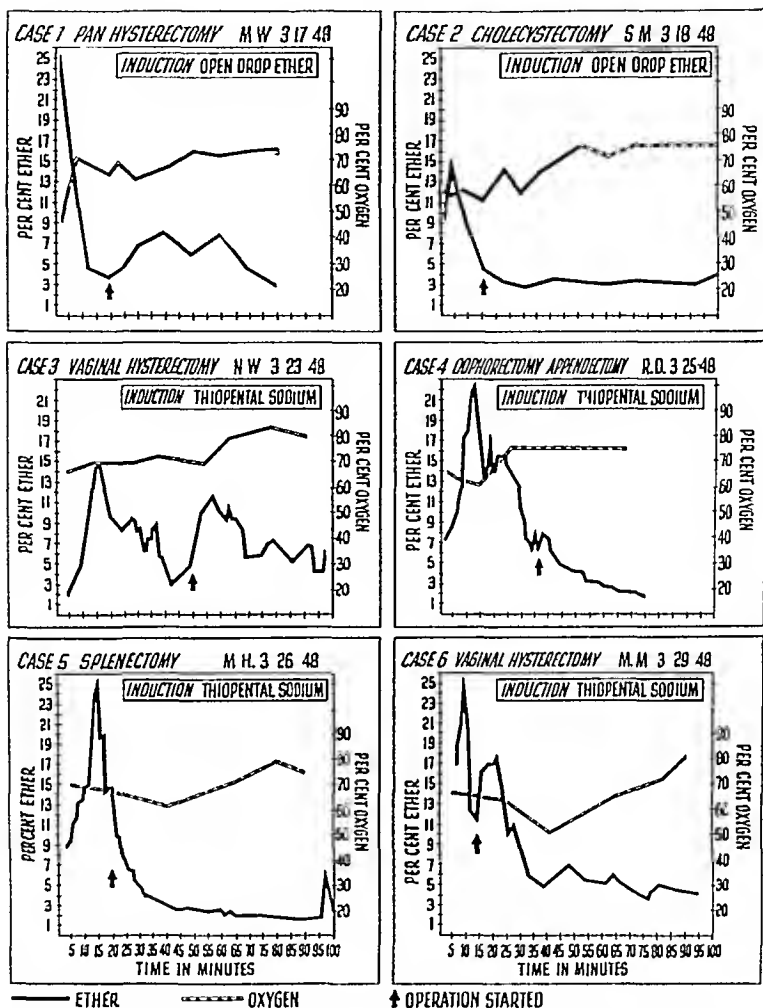


Fig. 9—Concentration of ether and oxygen in rebreathed gas during six surgical operations. Percentages are for the gas after removal of water and carbon dioxide.

7 to read the correct per cent of cyclopropane. The per cent of oxygen may be determined with the Pauling-Beckman oxygen meter, or by comparing the reading of the cyclopropane-free gas with that for air and calculating the oxygen content from the type of oxygen-nitrogen curve shown in Fig. 6.

Ternary mixtures of  $N_2O$ ,  $O_2$ , and  $N_2$  were also tested with the viscosity-effusion meter having a reference point of 62 cm with this reference point set for air. The results are given in Fig. 8. To determine the per cent of nitrous oxide during anesthesia, one should note the depression below the base line for air produced by the  $N_2O$ ,  $O_2$ ,  $N_2$  mixture and also determine the per cent of oxygen with the Pauling-Beckman oxygen meter. From these two results and the curves of Fig. 8 one can calculate the per cent of nitrous oxide.

Viscosity-effusion meter readings of ternary mixtures of ether-cyclopropane-oxygen and ether-nitrous oxide-oxygen are now being obtained. When such



surveys are completed we should be able to construct tables or nomograms to enable one to use the viscosity effusion meter to determine both ether and cyclopropane and ether and nitrous oxide when these pairs are used for anesthesia

#### CONCENTRATIONS OF ETHER AND OXYGEN DURING SURGICAL ANESTHESIA

Measurements with a viscosity effusion meter were made on the rebreathed gas from an anesthetic machine during a large number of surgical operations where ether was the only volatile anesthetic used. For the ether determinations the reference point was set with the ether free gas. Oxygen determinations were run on the ether free gas with the reference point set with air. Fig 9 gives the results for six of these cases, which illustrate some of the variations observed. It will be noted that, following the induction period, the concentration of the ether during the remainder of the operation ranged from 2 to 3 per cent for Case 5 to 5 to 7 per cent for Case 3. In all of the cases the oxygen content of the rebreathed gas was high, ranging from 70 to 80 per cent during most of the operation.

Results with a much larger series of ether anesthetics will be presented in a separate paper.

#### REMARKS

The particular sizes of capillary and orifice which we have employed were chosen to give a small flow of gas and to produce a manometer depression of about 1 cm per cent of ether. Here the capillary furnishes a little over 7 per cent of the total resistance to gas flow, the remainder being in the orifice assuming that the effective pressure drop across the orifice is 40 cm of mercury. The rate of gas flow may be varied, as desired, by varying the diameter of the orifice. Our experiments indicate that if the fraction of the total resistance to gas flow contributed by the capillary is kept constant the manometer reading for a given gas mixture will be the same, and, therefore, the same scale may be used for all instruments having this ratio of resistance between capillary and orifice. In other words, any instrument with a reference point of 52 cm of oil (sp gr 0.827) should give the readings shown in Figs 5 and 6. If one desires a higher or lower, manometer reading for the reference point this can be produced by varying the length or diameter, of the capillary. The effect will be to increase or decrease, the scale divisions, the change in scale divisions being proportional to the change in reference point manometer reading. While our data in Figs 5 and 6 should enable one to construct a scale for any instrument we suggest that each instrument be calibrated with a few concentrations of anesthetic gas to be read with it.

A rise in temperature slightly increases the manometer level for a given gas because of an increase in viscosity and a decrease in density. This rise for the 52 cm reference point is about 1.7 mm of oil per degree. Since most operating rooms are kept at a fairly constant temperature and the reference point is occasionally checked, and adjusted, if necessary, any error due to temperature changes will be insignificant.

The manometer drop for ether in nitrogen or oxygen is greater than the calculated drop, assuming that the viscosity and density of the mixture are strictly additive for the ratio of gases used. Tests with certain mixtures indicated that the deviation is due to altered viscosity. Various investigators<sup>23</sup> have reported that the viscosity of certain binary gas mixtures deviates from that calculated on a simple additive basis. Fortunately, this deviation is about the same for ether-nitrogen and ether-oxygen so that varying the oxygen-nitrogen ratio makes very little difference in the manometer drop for ether.

Our methods for preparing and storing specific concentrations of anesthetic gases in nitrogen, oxygen, or nitrogen plus oxygen have been described in detail because some other workers have reported considerable difficulty in these matters. Thus Haggard<sup>24</sup> states: "In carrying out the work presented in this series of papers, the author has been repeatedly confronted by a situation similar to Spence's. Carefully prepared ether-air mixtures have given an analysis of almost exactly one-half the calculated figure. A prolonged and unsuccessful effort has been made to account for the discrepancy in the results."

Using the methods described in the preceding pages, our ether mixtures gave analytic figures which agreed almost perfectly with the calculated values.

We have not yet tested some of the newer homologues of diethyl ether but there seems to be no reason why this apparatus may not be used for the determination of these compounds in mixtures with air or oxygen.

#### SUMMARY

1 This paper describes a simple apparatus for continuously indicating the concentration of certain anesthetic gases during surgical anesthesia.

2 The rebreathed gas from an anesthetic machine is passed through a capillary tube and then through a fine orifice. An oil manometer, bridging the ends of the capillary tube, registers the combined effect of changes in viscosity and density due to the concentration of the anesthetic gas.

3 The gas flow through the apparatus is about 100 c.c. per minute and the suction distal to the orifice needs only be maintained above about 400 mm. of mercury.

4 Since changes in oxygen concentration cause a small shift in the manometer base line, the entering gas is occasionally shunted through a tube of charcoal or other absorbent in order to adjust the base line.

5 The apparatus will accurately record the concentration of ether, cyclopropane, ethylene, and nitrous oxide when these are singly present in nitrogen or nitrogen plus oxygen. It will also indicate the approximate concentration of oxygen in nitrogen.

6 The procedure may be modified to determine the concentration of two of these anesthetic gases in rebreathed air.

7 Methods are described for preparing specific concentrations of anesthetic gases in nitrogen or nitrogen plus oxygen. For storing these mixtures without appreciable loss, a flexible aluminum gas bag was developed.

8 Readings obtained with the instrument are presented showing the concentration of ether and oxygen in the rebreathed gas during six surgical operations.

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# A MODIFIED LOEFFLER'S MEDIUM FOR CULTIVATING CORYNEBACTERIUM DIPHTHERIAE

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SINCE Loeffler<sup>1</sup> first published his formula for cultivating *C. diphtheriae*, numerous modifications of his medium have been reported. In 1932, Pai found that a coagulated egg medium supported growth of the *Corynebacterium* for all diagnostic purposes. Pai's medium had the disadvantage that a great many surfaces were irregular and pitted with air holes, with resultant losses of media batches.

For the past several years a modified Loeffler's medium, with incorporated egg, has been used successfully in this laboratory. This medium is superior to the routine Loeffler and the Pai media both in the cultivation of *C. diphtheriae* and in the demonstration of chromogenesis. Because of the many requests which we have received for our formula, it was decided to submit it for publication.

## EXPERIMENTAL

In the study of Pai's medium it was thought that the growth-promoting factor supplied by egg was a sulfur derivative. However, incorporation of sulfur-containing amino acids into our routine Loeffler's Trypticase medium<sup>3</sup> showed no significant additional growth promotion. Accordingly, trials were made in which whole fresh egg, previously beaten for about five minutes with a rotary egg beater, was added in varying quantities to the Loeffler medium.<sup>3</sup> The media were then inoculated with test organisms. Best results were obtained with a ratio of one whole fresh egg added to 1 liter of medium.<sup>3</sup>

## THE FORMULA

Trypticase (Peptone)*	20 Gm
Salt	5 Gm
Dextrose	10 Gm
Water	500 cc
Heart infusion broth (double strength)	500 cc
Whole egg (well beaten)	1

Ox serum was added in the ratio of 3 parts to 1 part of the broth formula. The pH was then adjusted with 2 per cent dipotassium phosphate, about 30 milliliters. It is important to obtain a pH of 7.8 to 8.0 before inspissating. The final pH, as determined in the water of syneresis in the bottoms of the tubes after inspissating, should be pH 7.6 to 7.8.

From the Bureau of Laboratories, Baltimore City Health Department.  
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\*Baltimore Biological Laboratory's product.

## COMPARISON WITH ROUTINE MEDIUM

Microscopic observations of approximately two hundred smears made from virulent and avirulent strains grown on the modified medium, in comparison with the routine medium,<sup>3</sup> showed the metachromatic bodies to have an intensified color and to be more brilliant and more sharply defined when stained with Loeffler's methylene blue. The large bizarre types A, B and C of Westbrooke<sup>4</sup> were markedly differentiated as were the polar and bipolar bodies.

The growth phase was enhanced. Perceptible growth is generally observed on the new medium and not on the routine at the end of four or five hours of incubation at 35° C. Smears made from these young cultures showed even more marked chromogenesis than described.

The modified medium, prepared as described is now used routinely in this laboratory. A dehydrated form is also commercially available.\*

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## AN EXPANSILE NEEDLE FOR THE INTRODUCTION OF INTRAVENOUS CATHETERS

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CARDIAC catheterization usually entails the sacrifice of a peripheral vein. During the course of the procedure, a peripheral vein is isolated surgically, incised for the introduction of the catheter, and finally ligated. A wide-bore needle has been employed to pass the catheter into the vein, but its use necessitates a small catheter and a large vein. A split needle† capable of being expanded following its entry into a vein makes it possible to insert wide-bore catheters through the puncture opening, minimizes surgery, and permits subsequent reutilization of the same vessel. The manipulations incident to the passage of the catheter are reduced to those involved in venipuncture.

The needle (Figs 1 and 2) consists of three parts: an outer split shell, a shaft, and a central stylet. The shell consists of two halves joined by a screw device to permit retraction from, and approximation to, the inner shaft. Its proximal end is adapted to receive the end of a syringe barrel. The shaft is a fine-bore, thick-walled needle with sharp tip, to which the shell is clamped by clockwise rotation of the screw. The fine stylet closes the lumen in the shaft.

In practice, the assembled needle is inserted through the incised skin into the vein lumen. The vein is not dissected free, its posterior attachments are left intact. Following insertion of the needle, its position in the vein lumen is verified by withdrawing the central stylet. If free blood flow is observed, the stylet is replaced, the shaft is withdrawn, the vein puncture opening spread by rotation of the screw, and the catheter inserted. The catheter is then advanced as the needle shell is withdrawn. At the termination of the procedure, following removal of the catheter, bleeding is usually minimal and readily controlled by pressure and Gelfoam.

The needle has been used successfully a number of times in dog and man for the introduction of cardiac catheters. The external jugular vein in the dog has been catheterized at weekly and semiweekly intervals. Gross examination has revealed scarring at the site of previous puncture with patency of the vessel lumen above and below the scarred zone.

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From the Cardiovascular Department, Medical Research Institute, Michael Reese Hospital. The Department is supported in part by the Michael Reese Research Foundation. Received for publication Feb. 3, 1949.

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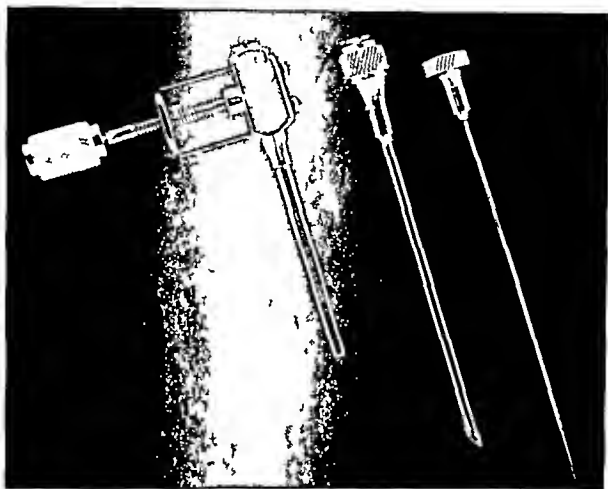


Fig 1

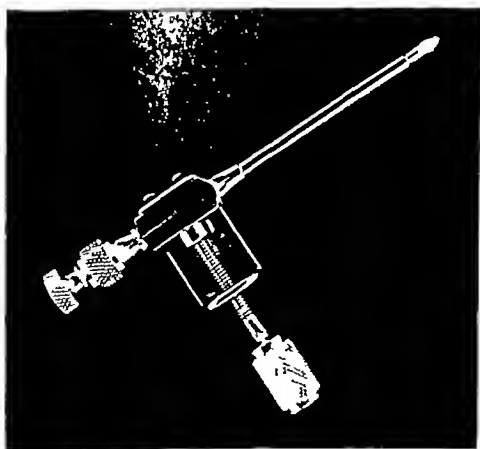


Fig 2

The residue from the acid extraction was suspended in 1 ml of water and then 4 ml of 95 per cent alcohol were added. The suspension was thoroughly mixed and centrifuged, and the supernatant was decanted. The residue was washed four times with 5 ml of 95 per cent alcohol and then three times with 5 ml of a boiling ether alcohol mixture. The supernatant and washings were collected and made up to 25 ml with alcohol. This fraction contains the ether alcohol soluble phosphorus and consists chiefly of phospholipids (Fraction 2).

The residue from the ether alcohol extraction was suspended in 2.5 ml of 1N KOH and the mixture incubated for twenty hours at 37° C. To the resulting clear solution, 0.6 ml of 6N HCl and 3 ml of 5 per cent trichloroacetic acid were added. The precipitate formed was separated by centrifugation. The supernatant contains the pentose nucleic acid and phosphorus from the phosphoprotein. This was decanted and made up to 10 ml with distilled water (Fraction 3).

The desoxyribonucleic acid remains with the residue and was brought into solution by boiling with 5 per cent trichloroacetic acid. The residue was separated and discarded. The supernatant was made up to 10 ml and designated the desoxyribonucleic acid fraction (Fraction 4).

Duplicate 0.5 ml aliquots of each fraction were dried under an infrared lamp and the phosphorus was then determined quantitatively in each case, using the method of King.<sup>7</sup> Counting of the radioactivity was done with a thin walled mica window counting tube and standard sealing assembly. The number of counts per 100 Gm, divided by the milligrams of phosphorus per 100 Gm is designated the "specific activity" of the fraction.

A series of *in vivo* counts over the tumor sites were also made on each patient at varying intervals after administration of the test dose. These counts were made with a standard gamma tube and sealing circuit. *In vivo* counting was surprisingly successful despite the fact that P<sup>32</sup> emits only a high energy beta particle (1.7 mev).

## RESULTS

Table I shows the quantitative uptake of P<sup>32</sup> by tumor as compared with normal brain tissue in ten subjects with intracranial tumor. Table II shows the fractionation of tissue phosphorus in four subjects studied by this technique. It is clear from these data that there has been an increase in concentration of the phosphorus in the tumor tissue in every instance as compared with the normal brain, the ratios vary from 1.6:1 to 29:1. The highest concentration obtained after intravenous injection is 19:1 in subject CA, in which case the white matter appears to be slightly less active than the gray matter. In general there appears to be a rough correlation between the phosphorus uptake and the anti-

TABLE I

SUBJECT	DOSE (MC)	INTERVAL TO BIOPSY	DIAGNOSIS	COUNTS/ MIN/GM TUMOR	COUNTS/ MIN/GM NORMAL	RATIO	ROUTE
AW	5	7 days	Control	4,500	4,500	1:1	Local
AA	5	45 hr	Glioblastoma M	3,180	770	4.2:1	Carotid
RG	5	96 hr	Glioblastoma M	4,770	162	29:1	Local
CA	5.5	2 hr	Melanoblastoma	2,266	(W) 122 (G) 146	19:1 16:1	I V
JP	1.6	48 hr	Glioblastoma M	1,006	474	2.1:1	I V
IR	10	15 hr	Glioblastoma M	3,200	245	13:1	I V
MK	10	2 hr	Cranio-pharyngioma	13,618	None taken		Oral
SO	10	18 hr	Meningioma	25,075	5,288	4.7:1	I V
LR	10	18 hr	Glioblastoma M	450,870	(W) 39,960 (G) 33,146	11:1 13:1	I V
SK	10	24 hr 11 days	Oligodendroglioma	37,950 58,460	21,290 36,800	1.8:1 1.6:1	I V

W White matter

G Gray matter,



TABLE II

	FRACTION	1 IN MC /100CM		COUNT/100 GM TISSUE x 10		SPECIFIC ACTIVITY	
Subject MK Cranopharyn- gioma	1	84.2		1.86		15.3	
	2	56.1		7.54		--	
	3	19.4		0.04		3.88	
	4	28				0.014	
		TUMOR	NORMAL	TUMOR	NORMAL	TUMOR	NORMAL
Subject SO Meningioma	1	67.0	40.0	11.250	2.80	1.78	7.0
	2	62.0	187.0	7.43	1.03	1.20	0.57
	3	40.4	18.1	6.05	1.27	1.50	6.9
	4	39.1	9.14	345	0.18	0.88	2.21
Subject IR Glioblastoma multiforme	1	72.0	7.7	280.0	14.9	38.8	2.78
	2	50.1	908	121.0	2.26	15.1	0.109
	3	31.1	16.0	47.7	22.8	15.3	14.25
	4	22.1	4.7	2.17	0.0	0.08	--
Subject SK Oligodendro- glioma (24 hr) (11 days)	1	68.4	10.0	79.4	18.8	5.77	3.36
	2	57.5	19.4	5.4	4.9	0.94	0.25
		7.5		12	11.5	16.4	5.7
	4	9.48	4.97	1.36	1.6	1.43	3.22
	1	4	14	16.8	8.35	0.50	1.27
	2	8.5	17.1	10.91	5.35	1.87	0.31
	3	15	19.5	7.6	6.67	4.08	3.30
	4	19	8.5	2.64	0.96	1.96	1.13
Fraction 1 acid soluble P Fraction 2 alcohol soluble P Fraction 3 ribonucleic acid P Fraction 4 desoxyribonucleic acid P							

pated metabolic activity of the tissue as judged on the basis of the degree of malignancy. In the fractionation studies likewise concentration by the tumor exceeds that by normal brain in every instance but one (Subject SK, Fraction 4). Concentration of phosphorus in Fraction 4 is lower than the others, as would be expected, but rises with the passage of time as indicated by the second biopsy in Subject SK. The lack of any concentration in Subject AW (Table I) is due to the fact that the tumor had been removed surgically several days before administration of the phosphorus and the specimen was obtained from the area immediately underlying the area of removal which was subsequently shown histologically to be free of tumor tissue.

In vivo counts were only moderately successful with the apparatus used thus far. Increased counts over the tumor sites were recorded quite consistently but the ratios of increase were small and the localizing value of this technique for diagnostic purposes is to be determined.

# DISCUSSION

A period of several years would be necessary to evaluate properly any therapeutic effect that may be obtained from the radiation derived from this isotope and such information is not available at the present time. To anticipate a beneficial response, selective absorption of the isotope in high concentration by the tumor tissue is a prerequisite but, in addition, a high stability of its incorporation in cell protoplasm with a low turnover rate is also desirable. In this connection, phosphorus incorporated in nucleoprotein is known to be more stable than that in the phospholipid or ester fractions. For this reason our

interest centers chiefly about the more stable intranuclear and cytoplasmic phosphorus of Fractions 3 and 4 (vide supra). Desoxyribonucleic acid (fraction 4) occurs chiefly in the nuclei of cells and is believed to serve an important function in cell division. Ribonucleic acid (Fraction 3) is a constituent of cytoplasm and nucleoli of cells and appears to play a dynamic part in metabolic activity, especially in those cells in which active protein synthesis is occurring. There is some reason to believe<sup>8</sup> that the desoxyribonucleic acid of the nucleus is formed at the expense of cytoplasmic ribonucleic acid, and since the rate of this reaction correlates with the rate of tissue growth,<sup>9</sup> it may be inferred that the nuclear fraction is built up during mitosis. Blues, Tracy, and Cohn<sup>10</sup> have shown that desoxyribonucleic acid phosphorus is very stable in the nongrowing organ. Its rate of turnover is surprisingly slow, especially in contrast to that of the ribonucleic acid fraction. These same authors have demonstrated the rate of desoxyribonucleic acid phosphorus accumulation in experimental hepatoma to be intermediate between resting and regenerating liver. If these data are applicable to malignant brain tumors, the higher metabolic activity of growing neoplastic cells might be expected to show a greatly augmented phosphorus metabolism, leading finally to a sharp rise in the more stable nuclear fraction. Based upon the same reasoning, it might also be anticipated that repeated administration of  $P^{32}$  to maintain a constant supply of inorganic isotopic phosphate in the metabolic pool would lead to the accumulation of a higher desoxyribonucleic acid phosphorus ( $P^{32}$ ) concentration than could be obtained from a single dose of the isotope. Thus, on theoretical grounds there is some justification for the administration of thirty doses of 333 microcuries each, in preference to a single dose of 10 millicuries, if a sustained radiation effect for therapeutic purposes is to be maintained. This principle should be equally applicable to any rapidly growing malignant tumor by virtue of its high metabolic and growth rates. In any clinical use of  $P^{32}$  the capacity of normal, metabolically active tissues, especially bone marrow, to withstand the radiation will be the limiting factor. Additional investigation of this principle demands further attention.

#### SUMMARY

1 Inorganic phosphate containing  $P^{32}$  was administered parenterally to patients with malignant intracranial tumors.

2 Biopsy material was obtained, after which, by means of standard chemical techniques, the ratio of concentrations of radioactivity in tumor and normal brain tissue was shown to rise to as high as 19:1.

3 Chemical fractionation of tissue phosphorus showed a small but significant concentration in the nucleoprotein of both cytoplasm and nucleus. The implications of these findings are discussed.

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# TISSUE RESPONSES TO PHYSICAL FORCES

## II THE RESPONSE OF CONNECTIVE TISSUE TO PIEZOELECTRICALLY ACTIVE CRYSTALS

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### I INTRODUCTION

THE manner in which biologically active dusts produce fibrotic reactions in tissues has stimulated much thought and research. The development of this problem has been reviewed frequently, most recently by Machle.<sup>1</sup> There is general agreement among authors that present explanations cannot be reconciled with all experimental and clinical observations. The observations unexplained will be recalled later in this discussion.

The interrelationship between various endogenous and exogenous fibrotic stimuli has increased the complexity as well as the importance of this problem. It is recognized that the biologic activity of quartz dust and of tuberculosis is interchangeable and "symbiotically" additive.<sup>2</sup> Crystalline cholesterol and other "endogenous" substances bear similarly interchangeable relationships with quartz and the phospholipid of tuberculosis.<sup>3</sup> Fibrogenesis appears to be a common response to many stimuli. Indeed, fibrogenesis seems to be influenced more by biologic variation and the site of involvement than by the specificity of its stimulus.

Since different stimuli produce a common biologic response, it appears that apparently dissimilar fibrogenic stimuli might share some "common denominator." This notion is strengthened by the interchangeability of quartz and tuberculosis, and by the increased response produced by adding one to the other.

We have assumed that, if a living organism survives, its response to injury is purposeful. An examination of the functional qualifications of a reaction should, therefore, provide valid inferences as to the nature of the injury. Fibrous tissue has not been demonstrated to offer a barrier to the diffusion of substances in solution beyond that usually offered by the tissue it replaced. Fibrous tissue has not been shown to detoxify a toxin, neutralize a poison, or fix an antigen. While young fibroblasts are possibly related to phagocytosis, phagocytosis is hardly a functional defense against a "poisonous solution." It is apparent that a fibrous reaction is not able to offer functional defense against the "slowly soluble poison" which is considered by most investigators to be the mechanism of injury in silicosis.<sup>1</sup>

Fibrous tissue in normal physiology provides structural support against stress in a physical sense. Fibrous proliferation should, therefore, be stimulated by stress or the need for support. The osteoblast clearly responds along

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stress vectors as is so plainly shown in the architecture of long bones. Perhaps its parent cell, the fibroblast, is similarly responsive.

The architecture of the silicotic whorl conforms closely with the equations of force. This is clearly demonstrated by its similarity to the stress lines depicted with a magnet and iron filings. (This is illustrative and is not intended to imply that magnetic force is thought to be fibrogenic.)

Perhaps the notion that fibrous reactions are best equipped to confine stress is what led early observers to the "sharp point" view of silicosis. Were "sharp points" the functional stimulus, one would expect the reaction to stop when the points were blunted rather than continue beyond any apparent need.

## II. THEORETICAL CONSIDERATIONS

As has been stated already, fibrous tissue proliferations function as supportive components in normal body economy and are best qualified to fill this role in disease as well. It also has been emphasized that even as fibrous tissue responds along stress vectors in normal growth, it also distributes itself architecturally in close conformity with Newton's "equations of force" when it forms the predominant reaction to an injury. These observations, though elementary, clearly demand that a careful search for a factor of stress in the physical sense be made wherever fibroblasts proliferate in a superfluous manner.

As stated in a preliminary paper<sup>1</sup> it has been observed that there is a direct relationship between the ability of a dust to produce a fibrotic reaction in tissue and the crystal symmetry of the dust. Asymmetrical (piezoelectric) dusts are observed to be fibrogenic whereas symmetrical (nonpiezoelectric) dusts are benign.

Piezoelectricity is that property of certain solid materials which effects when conditions are proper a transference between electrical and mechanical energy states. This transference operates in either direction. As an example, it is this property of quartz which permits the use of quartz crystals in phonographs where energy is converted from a mechanical vibration produced by the record's groove to the electric current which energizes the loud speaker.

It is necessary to present some definitions of terminology to be used in the discussion of the piezoelectric phenomenon as it relates to biology.

*Piezoelectricity*—Piezoelectricity is the property possessed by some crystals, of developing electric charges when compressed or extended in particular directions. Conversely when a potential difference is applied to suitable points on such a crystal, it expands or contracts. Piezoelectric properties can occur in all crystals lacking a center of symmetry.

It may be stated with authority<sup>6a</sup> that molecularly symmetrical crystal material cannot possess piezoelectric properties. When matter exists in a crystal which has no point of central molecular symmetry it is then potentially qualified as piezoelectric.

All crystals exist with a molecular orientation which classifies them in one of thirty-two classes. Only those crystals belonging to classes lacking a

central point of symmetry can be piezoelectric. In a preliminary report<sup>4</sup> it was shown that, where data are available, benign dusts uniformly belong to symmetrical crystal classes, while fibrogenic dusts belong to asymmetrical classes. A few special instances of dusts requiring further comment as apparent exceptions will be considered later. Approximately 10 per cent of minerals existing in nature have molecular orientation compatible with the property of piezoelectricity.

One must consider what sources of energy might activate the mechanism if the fundamental stimulus in fibrous reactions is considered as a transformation of noninfluential energy states through piezoelectricity to forms of energy which comprise biologically influential stress. There are several possible sources of energy which suggest themselves:

- 1 Electromagnetic fields may exist in nature continuously or in "bursts" in sufficient concentration and at proper frequency to produce mechanical distortions of receptive particles within tissue.

- 2 Electropotential reversals occurring in normal physiology could produce momentary physical distortion in receptive particles.

- 3 Fluctuant pressure variations secondary to pulsant circulation, respiration, cough, strain, position change, etc., might induce polarity and electric current on a receptive particle.

- 4 Within a deposition composed of many packed particles, either response in one particle might induce the opposite response in any neighboring deposit within its sphere of influence.

- 5 Thermal variations within the animal can produce variations in electropotential and distortions in shape in receptive crystals (secondary piezoelectric effect).<sup>6b</sup>

There have been many clues recognized by biologic observers that lead one to believe the conventional description of a biologic environment is incomplete. This topic will be discussed later in the light of intensity and frequency of the energetic field within which the host is situated.

Purely for test purposes, a number of "postulates" for a fibrosive dust have been conjectured. These are listed at this point as they summarize in a practical manner the theoretical considerations so far presented.

*"Postulates" for a Biologically Fibrosive Dust —*

- 1 *Relative insolubility* in body fluids to allow particles to remain in situ long enough to be effective.

- 2 *Asymmetrical crystal class* compatible with piezoelectric properties.

- 3 *Electrically nonconductive* as conductivity will "short circuit" and nullify the piezoelectric effect.

- 4 *Proper particle size*, small enough to be encircled by existing concentrations of force fields, yet not so small that they be "out of reach" of existing electromagnetic frequencies or so small that their induced strain might be "subfibrogenic."

- 5 *Sufficient particle concentration* per unit mass of tissue so as to allow sufficient magnitude of physical effect to stimulate the reaction, or to allow

reciprocal physical influence between the unit particles, thereby permitting the deposition to become a system operating in resonance

### III. EXPERIMENTAL PROCEDURE

The purpose is (1) to determine if molecular asymmetry and piezoelectricity are fundamental in the biologic activity of dusts, and (2) to determine the sources and forms of energy involved in the energy transferal mechanism

**PURPOSE 1**—To determine if molecular asymmetry and piezoelectricity are fundamental in the biologic activity of dusts

*General Statement*—Rats and rabbits were used in the experiments. The materials to be tested were first ground to desired size. Introduction of the material into the animal's body was done in different ways. In the early experiments the ground material was suspended in physiologic salt solution and sterilized. It was then injected into the peritoneal cavity with a modified syringe.

Because of difficulties due to plugging of the needle of the syringe, and because in some experiments it was desirable to use larger sized particles, this first method was modified. A small incision was made in the ventral abdominal wall, a small sterile funnel inserted, and dry sterile powder introduced through the funnel. The funnel was washed down with a small quantity of sterile physiologic salt solution. The quantity of material introduced in this way was always 100 milligrams. The incision was then closed with a skin clip. After closing the incision, the abdomen of the animal was gently massaged to distribute the material in the abdominal cavity.

In some animals the material was introduced into the trachea. The trachea was exposed and material forced down the tube toward the lungs with a modified syringe. Fifty milligrams was the quantity always introduced by this method.

In another group of experiments the sterile material was introduced by intravenous injection. Rabbits were used in all of these experiments. By one or more injections a total of 1 Gm. of material was injected into a vein of the ear.

For purposes of comparison, six animals were prepared with powdered quartz. The animals were sacrificed at varying intervals of time and microscopic sections prepared. The well known typical reaction to quartz is shown in Fig. 3.

*Wulfenite (Lead Molybdate), Crystal System—Tetragonal, Crystal Class—Tetrahedral*—This material was the major test substance used in preliminary experiments which were reported.<sup>4</sup> Unfortunately, the supply of material on hand became exhausted and material of identical x-ray diffraction pattern has been thus far unobtainable. The reason for the selection of this material and the description of the experimental results were given in the preliminary report.

Forty-seven rats were prepared by injecting suspensions of wulfenite into the peritoneal cavity. The animals were sacrificed at different intervals from one to fourteen weeks. Four of the rats are still living. The average length of time the animals were allowed to live was thirty-four days.

Wulfenite had caused extensive fibrogenesis in animals which were sacrificed after seven days. Large numbers of fibroblasts were accumulated around the particles of wulfenite. There was a tendency to form whorls. In some instances, younger connective tissue cells were present. These were irregular stellate cells surrounded by a homogenous intercellular substance. There was a tendency to the formation of collagenous fibers in animals in which the experiments were continued for a longer period of time. See Fig. 5.

Two of the rats were killed by exsanguination at six weeks. The blood was allowed to dry by evaporation and the residue was spectriographically analyzed. No lead or molybdenum was present. As the technique employed is sensitive to amounts of molybdenum as low as one part in a billion, and for lead, one part in one hundred million, it was apparent that solubility could not explain the reaction produced.

*Barium Titanate, Crystal System—Cubic, Crystal Class—Undetermined*—This synthetic material has recently excited much interest in the physical fields and the ceramic industry. It possesses a very high dielectric constant, and its piezoelectric coefficients are far in excess of those possessed by quartz.<sup>8</sup> The material used in this study was analyzed by an independent metallurgical laboratory and was found to contain less than 0.2 per cent silica. The material was insoluble in any pH conceivable in animal tissue. The material was received in a particle size below  $10\ \mu$  and no ball milling was necessary.

Twenty-five rats were injected intraperitoneally in the usual manner with suspensions of the material. These animals were sacrificed at periodic intervals from one to eight weeks with an average of twenty-four days' duration for the group.

*Results* By the end of the first week, the barium titanate depositions were noted to have become arranged in well-defined whorls, and a fibroblastic, relatively avascular reaction was already well defined. Giant cells and monocytes were not as numerous as with some of the other materials tested. As the time interval progressed, the whorls became increasingly fibrotic and collagenic. Based on experiences with quartz, it was observed that barium titanate was highly active in producing fibrogenesis in tissue. See Fig 10.

Four rats from the six-week duration group were killed by exsanguination. The blood obtained was allowed to dry and the residue subjected to spectriographic analysis. No barium or titanium lines were found. This analytic technique is qualitatively sensitive for barium or titanium when traces of either element are present in as low as one part in a billion for titanium and one part in one hundred million for barium. It is, therefore, safe to say that there was no solution of the barium titanate from the sites of deposition. These data are advanced to answer any "chemical solution" explanation for the fibrous reaction.

Nine rabbits were prepared by injection of a full gram of barium titanate in divided doses into the ear veins. This dose was accomplished in less than a week in all cases. These rabbits were sacrificed from two weeks to seven weeks after the completion of the preparation. Again, excessive fibroblastic reactions were noted at the site of barium titanate depositions. Interstitial pulmonary fibrosis, mesenteric fibrosis, and hepatic cirrhosis conforming to the distribution of the crystal were apparent in all animals.

It was observed that the reaction to this material in rabbits prepared intravenously was the same as that observed in rats.

*Tourmaline (Aluminum-Boron Silicate), Crystal System—Trigonal, Crystal Class—Hemimorphic Hemihedral*—This silicate is of historic interest be-



cause it is the substance in which the property of piezoelectricity was first described. It is of additional interest in that it does not have true right and left forms such as seen in quartz.

Eight white rats were prepared in the usual manner by intraperitoneal injection of a suspension of powdered tomahline.

Four of the eight white rats were found dead in the fifth week of the experiment. Death had occurred over a week end and all were found on the same morning. They were all too far decomposed for histologic preparations. Gross fibrosis was present in all instances. Food and fresh water remained unconsumed and the cause of death was not determined. The experiment is being repeated in larger numbers to ascertain any possible significance of these deaths.

Two animals were killed at three weeks, and two at four weeks. Fibrotic reaction to the material was very excessive and intercellular collagen deposition was well developed by the end of the fourth week. See Figs 11, 13 and 15.

*Berlinite (Aluminum Orthophosphate), Crystal System—Trigonal, Crystal Class—Enantiomorphous Hemihedral*—Berlinite is aluminum orthophosphate which is physically and crystallographically isomeric with quartz. Our material was grown out of solution by the originators of a method developed to synthesize crystals for physical purposes. The material is soluble in acids, but insoluble in neutral solutions. The similarity in crystal structure between berlinite and quartz is illustrated by the striking similarity in their x-ray diffraction patterns. See Figs 1 and 2.

Eight white rats were injected intraperitoneally in the usual manner, using a ball milled powder of requisite particle size. These animals were sacrificed as follows: four at two weeks, two at three weeks, and two at five weeks.

At two weeks, a well developed fibrotic response was observed with considerably more monocyte and giant cell reaction than was seen with quartz or barium titanate. This monocyte response was noted in our studies whenever materials of greater relative solubility were used.

As time elapsed, the fibrous reaction advanced and became more mature. By five weeks, there was considerable intercellular collagen deposited. See Fig 4.

*Aluminum Phosphate (Reagent Grade)*—This material was selected originally for a control. It was determined to be amorphous by x-ray diffraction prior to preparation for injection. The material is insoluble in neutral solvents but is soluble in acid solvents.

Six rats were injected intraperitoneally in the usual manner with suspended aluminum phosphate. These animals were killed in pairs at three weeks, five weeks, and six weeks.

A spectacular fibroblastic reaction was observed at three weeks which increased with time, and by six weeks fibrous nodules as large as 1 cm in diameter were distributed throughout the peritoneum. Many giant cells were noted about the depositions of aluminum phosphate.

It was observed that, as time elapsed, fracture lines and facets appeared in the aluminum phosphate depositions which then resembled the beilinite deposits. The fibrous reactions were histologically similar to those seen with beilinite. This unexpected result from an intended control experiment will be considered in the discussion. See Figs 9, 12, 14, and 16.

*Cholesterol*—The crystal class of cholesterol is not certain, but, because of its known fibrotic activity when deposited in tissue in its solid crystal state, a series of experiments was included in this study. Commercial cholesterol in powdered form was x-ray diffracted and its crystal state was established.

Eight white rats were injected and were sacrificed at the following intervals: two at three weeks, two at four weeks, two at six weeks, and two at eight weeks. Several frozen sections of lesions were made in addition to the usual preparations, because xylol removed the cholesterol crystals leaving spicular spaces at the sites of previous crystal deposits.

The fibroblastic response was early and very active. Giant cell formation was prominent and similar to the reaction produced by aluminum phosphate. By eight weeks a mature nodule had developed. Inter cellular collagen formation was prominent. See Figs 6 and 8.

*Silicon Carbide (Carborundum), Crystal System—Hexagonal, Crystal Class—Holohedral*—This material was included as a control because it is not piezoelectric and is known to be nonfibrosive. Six rats were injected intraperitoneally in the usual manner. These animals were sacrificed in pairs at two, six, and eight weeks.

The reactions observed did not exceed simple foreign body encapsulation. See Fig 19.

*White Mica, Crystal System—Trichinic, Crystal Class—Prismatic*—Mica was included in this study as a control as it belongs to that group of silicates including talc and asbestos which produces variable biologic response.

Seven white rats were prepared intraperitoneally in the usual manner. Six animals were sacrificed at five weeks and one at six weeks.

Four of the animals were observed to show no reaction beyond simple foreign body encapsulation. Two showed a moderate fibrotic response, and the animal sacrificed at six weeks showed a well-developed reaction. See Fig 20.

*Fused Quartz and Ground Pyrex Glass*—Glass is a "super viscous fluid." Its viscosity prevents its becoming oriented molecularly into the preferred "low energy" crystal state.

Because it lacks crystalline structure, it possesses no piezoelectric properties. With time, glass may reassume crystalline orientation as is well known to glass cutters who will accept the risk in cutting new glass, but refuse to cut old glass which often shatters along crystal lines.

A sample of quartz crystal was fused and thereafter ground to a powder. A sample of Pyrex glass also was ground to a powder. Both materials failed to show a crystal pattern when examined by x-ray diffraction. The resulting

powders reduced to requisite size were injected intraperitoneally in the usual manner. Three white rats were used for each material. One animal of each group was killed at two, six and eight weeks.

No reaction beyond simple foreign body encapsulation was observed. See Fig 17 and 18.

**PURPOSE 2**—To determine the sources and forms of energy involved in the energy transferral mechanism.

It has been pointed out that fibrotic activity of a dust may be correlated directly with the physical properties of the dust crystal. Further clarification of the mechanisms involved becomes pertinent. It is important to determine whether electric charge or mechanical distortion produce the most active biologic reaction. A theoretically complete separation of the direct and converse piezoelectric reaction and of the secondary thermal reactions is impossible, but when conditions are controlled one of the other reactions may be made to predominate. To this end several experimental procedures were devised. These are briefly described and separate reports will present details of procedure and results.

**1 Mixed Metal Experiment**—When two metals which occupy dissimilar positions in the electromotive series are placed in an electrolyte a battery producing a flow of direct current results. The voltage of such a battery is a factor of the connecting electrolyte and of the degree of electromotive dissimilarity in the metals considered. As the same technique is employed in the procedures to be described the factor of "connecting electrolyte" may be ignored as a relative constant.

Particles of electromotively separated metals introduced into tissues and so "connected" by electrolytic tissue fluid would produce direct currents. The bimetal dipoles in such a system might become distorted in their electrical axes to conform with a static or magnetic field but the *preponderate* activity would be a manifestation of electric potential.

The electric currents produced with mixed electromotively dissimilar metallic dusts in tissue would differ from those produced by a tissue deposition of stressed piezoelectric crystals because the current flow would be continuous and direct rather than intermittent and alternating. Furthermore the battery produced would "run down" with time and use while the crystal remains potent for the life of the animal. The similarity however is sufficient to warrant biologic study.

Aluminum, iron and silver dusts were selected for study. Aluminum is strongly negative to silver and weakly negative to iron. Silver is strongly positive to aluminum and weakly positive to iron. The dusts were in each instance freshly suspended in weighed amounts before injection.

It was anticipated that strongly negative or positive dusts might form a battery when injected alone due to interaction with free metallic ions in tissue fluid. The single and mixed dust combinations studied are listed in

levels in accordance with the expected voltage (and biologic effect), the most active level being uppermost

First level	(Highest voltage)	Mixed Al and Ag
Intermediate level	Mixed Al and Fe Al alone	Mixed Ag and Fe Ag alone
Third level	(Little or no voltage)	Fe alone

In order to simplify presentation and avoid confusion, the results of "mixed metallic dust" experiments are reported separately. It may be stated here that galvanic currents stimulate fibrosis. See Fig 7. The rapidity and degree of fibrotic reaction is directly proportionate to the degree of electromotive dissimilarity between the metallic dusts selected for mixture. This is equivalent to stating that the reaction is proportionate to the amount of current produced. This is in agreement with observations made by others<sup>9</sup> working with large metallic plates inserted into tissue.

2 *Biologic Augmentation by Electromagnetic Fields*—Piezoelectric crystals of nearly uniform size possess fundamental frequencies for each of their several axes. At these frequencies, an alternating current produces a mechanical vibration with greatest efficiency. Similar crystal responses occur at several subharmonic and superharmonic alternating current frequencies.

It may be seen that there is a frequency range at which a piezoelectric dust of different size range may be expected to be energized. For some substances, as quartz, this range may be determined from known data. For other substances, an exciting frequency range may be determined by physical test means.

Several cages have been supplied with radio frequency energy in order to expose dust placed in a living tissue to various frequencies of energy fields. It was anticipated that, by so doing, a biologic response might be accelerated. This approach was, we believe, prematurely overemphasized in a preliminary report.<sup>4</sup>

This problem has been beset by many technical difficulties which are being resolved slowly. A detailed report will be submitted separately.

It may be stated that, in agreement with the preliminary report,<sup>4</sup> there is a definite increase in the rapidity and degree of fibrotic reaction to a fibrostatic dust when the prepared animal is exposed to oscillating fields of energy at a frequency known to produce piezoelectric responses in the same dust in vivo. This is supportive evidence that physical stress and motion are capable of stimulating fibrotic reactions.

Several other experiments testing the role of heat, motion, and electricity in fibrogenic reactions are in progress and will be reported separately.

#### SUMMARY OF EXPERIMENTS

1 Piezoelectric materials, including wulfenite, quartz, barium titanate, tourmaline, berilmite, and cholesterol, uniformly produce proliferative fibrous reactions when introduced as a finely divided dust into living tissue.

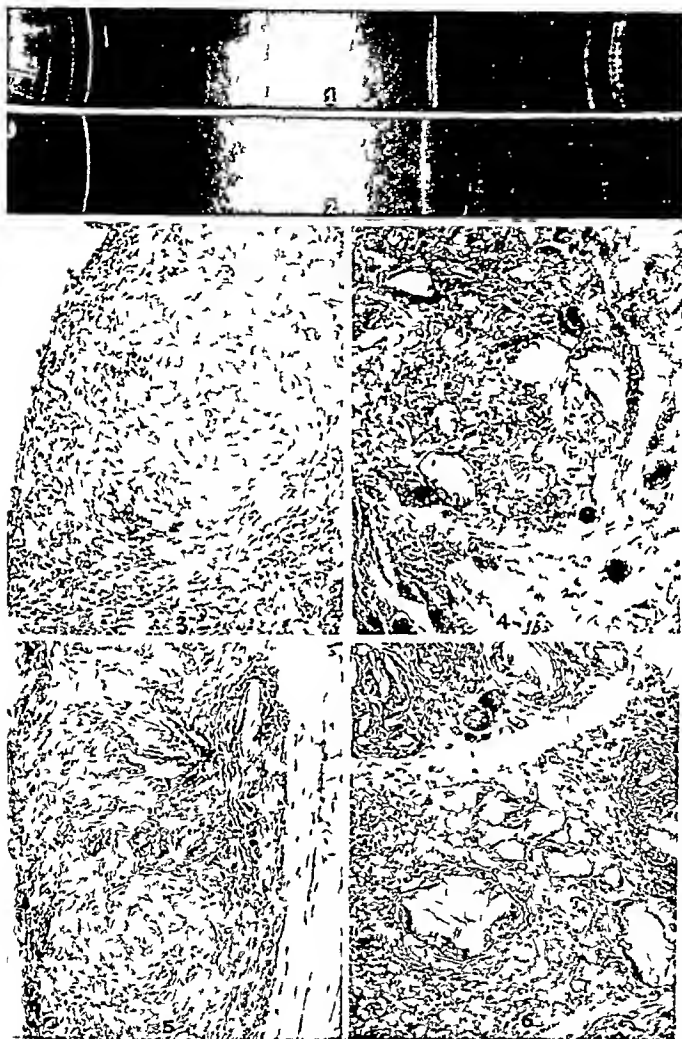


Fig 1—Monochromatic x ray diffractionogram of quartz powder technique  
 Fig 2—Monochromatic x ray diffractionogram of berlinite powder technique  
 Fig 3—Intraperitoneal nodule produced with powdered quartz in four weeks in a white rat (*Rattus norvegicus*) ( $\times 200$ )  
 Fig 4—Intraperitoneal nodule produced with powdered berlinite in two weeks in a white rat. ( $\times 200$ )  
 Fig 5—Intraperitoneal nodule produced with powdered wulfenite in four and one half weeks in a white rat ( $\times .00$ )  
 Fig 6—Intraperitoneal nodule produced with powdered cholesterol in six weeks in a white rat. ( $\times 200$ )

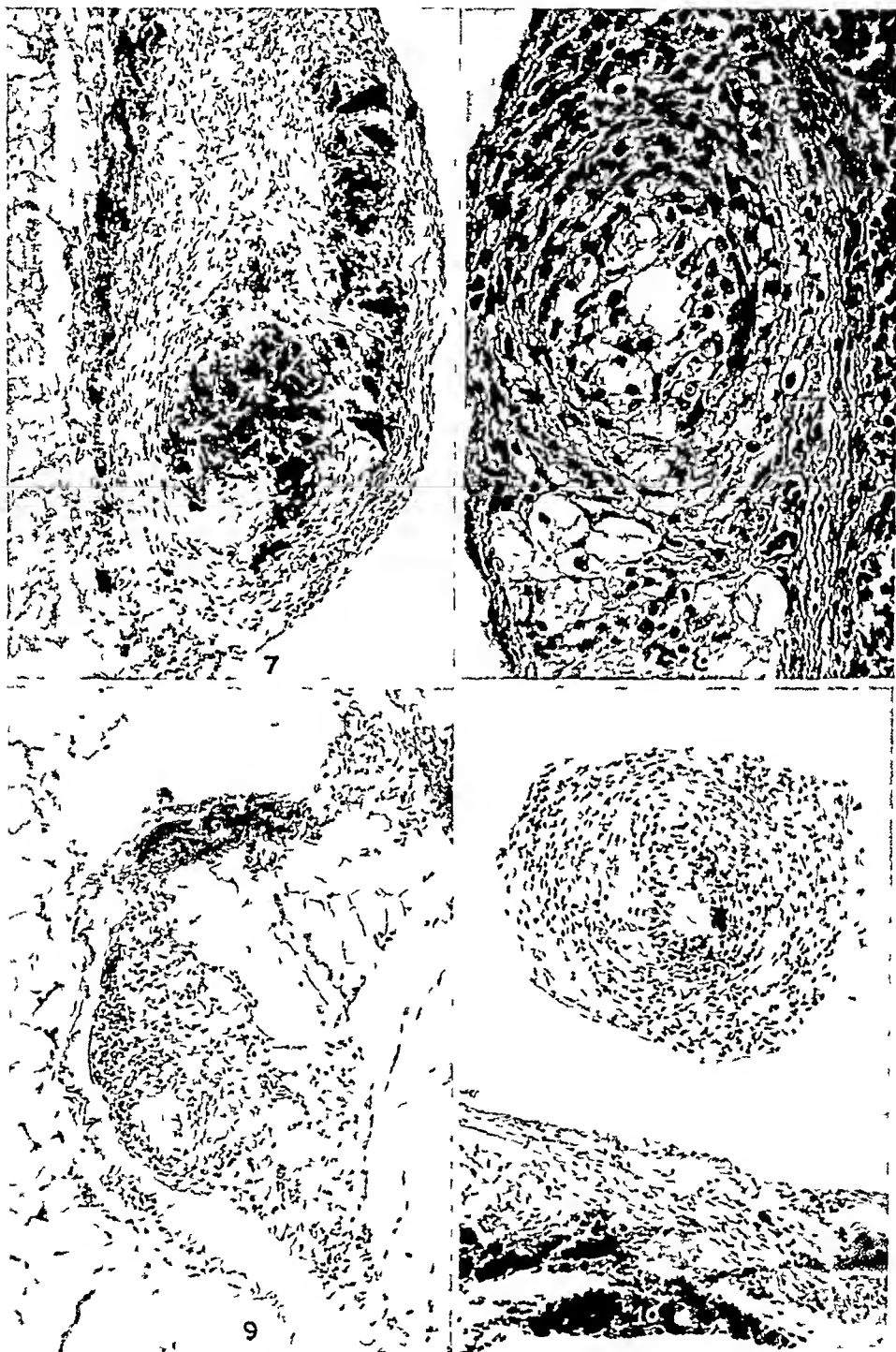


Fig 7—Intraperitoneal nodule produced with a mixture of powdered silver and aluminum in one week in a white rat. ( $\times 200$ )

Fig 8—Intraperitoneal nodule produced with powdered cholesterol in six weeks in a white rat. Foam cells similar to those seen in atherosclerosis are present. ( $\times 400$ )

Fig 9—Intraperitoneal nodule produced with powdered aluminum phosphate (reagent grade) in four and one-half weeks in a white rat. ( $\times 200$ )

Fig 10—Intraperitoneal nodule produced with powdered barium titanate in four and one-half weeks in a white rat. Crystals are present in the center of the nodule. ( $\times 200$ )

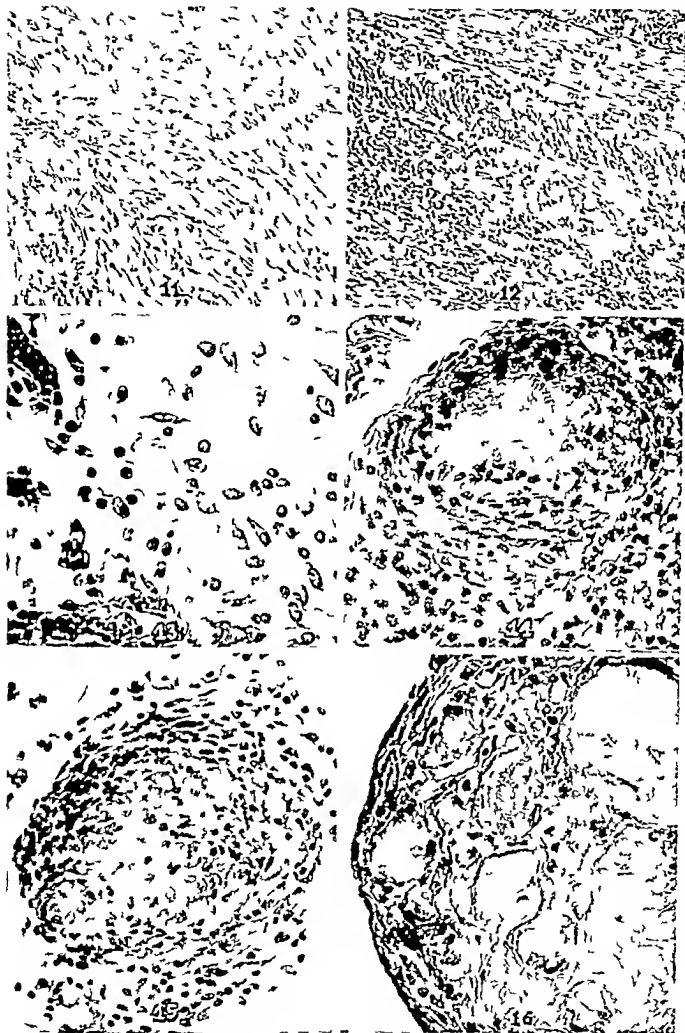


Fig 11—Intraperitoneal reaction to powdered tourmaline in four and one half weeks. Portion of a 1 cm mass attached to stomach. ( $\times 100$ )

Fig 12—Intraperitoneal reaction to aluminum phosphate (reagent grade) in five and one half weeks. Portion of a 1 cm mass attached to the gut. ( $\times 100$ )

Fig 13—Intraperitoneal reaction to tourmaline powdered in four and one half weeks high magnification. Young connective tissue cells and intercellular fibrils are present. ( $\times 400$ )

Fig 14—Intraperitoneal nodule produced by aluminum phosphate (reagent grade) in four and one half weeks in a white rat. ( $\times 400$ )

Fig 15—Intraperitoneal nodule produced with powdered tourmaline in three and one half weeks in a white rat. ( $\times 400$ )

Fig 16—Intraperitoneal nodule produced with powdered aluminum phosphate (reagent grade) in five and one half weeks in a white rat. Collagenous fibers are well formed. ( $\times 400$ )

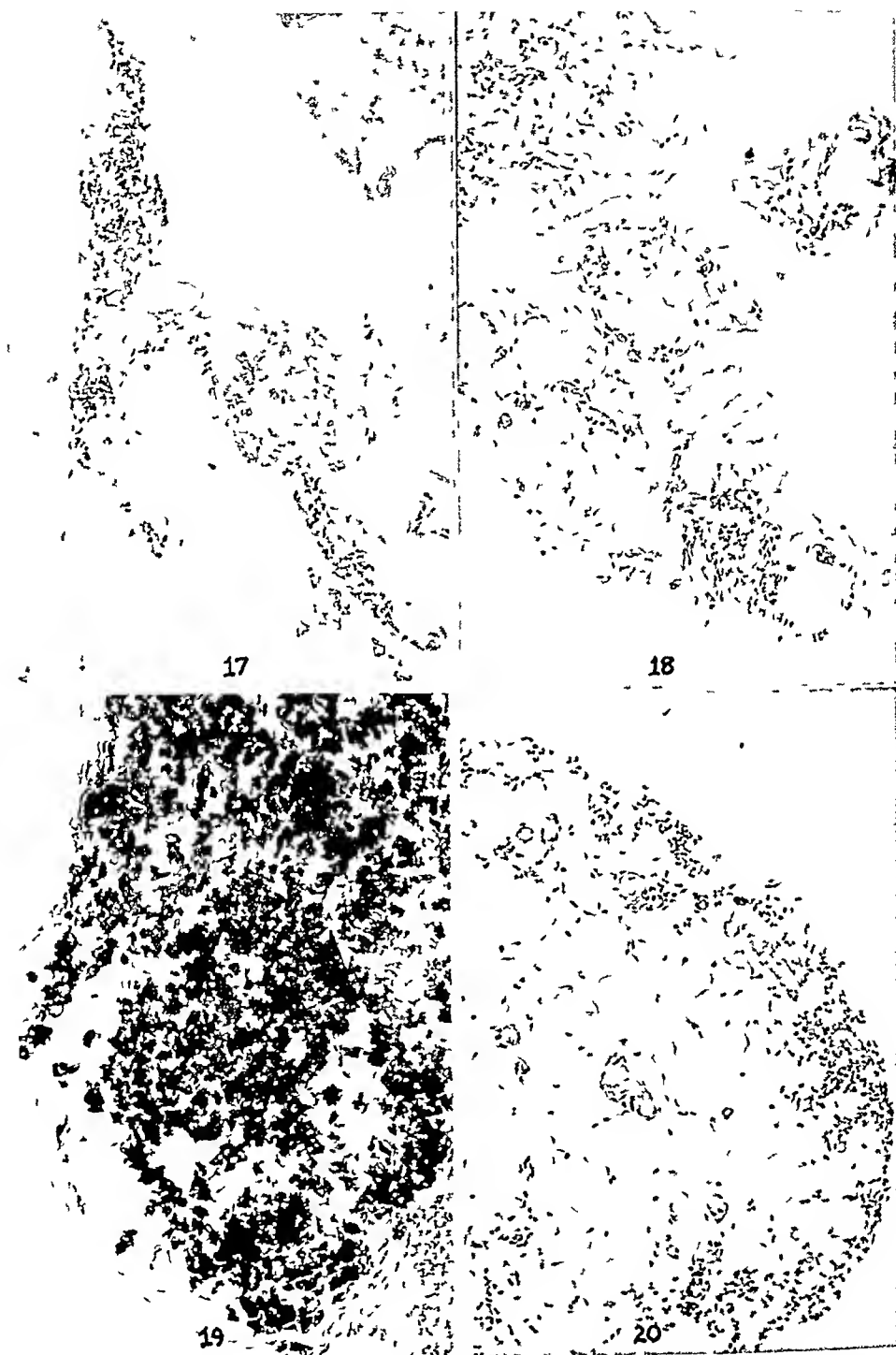


Fig 17—Intraperitoneal nodule produced with ground Pyrex glass in six weeks in a white rat. ( $\times 200$ )  
 Fig 18—Intraperitoneal nodule produced with fused quartz in six weeks in a white rat. ( $\times 200$ )  
 Fig 19—Intraperitoneal nodule produced with silicon carbide in six weeks in a white rat. ( $\times 200$ )  
 Fig 20—Intraperitoneal nodule produced with white silica in five weeks in a white rat. ( $\times 200$ )



2 Nonpiezoelectric control dusts including ground glass and fused quartz, silicon carbide and non produce only an encapsulation reaction when introduced into living tissues

3 Mica produces variable responses in living tissue

4 Aluminum phosphate was used as an amorphous powder as a control dust. Crystal forms appeared in the tissue reaction and the reaction was notably fibrotic. Aluminum phosphate with water of crystallization occurs in forms which are crystal isomers of quartz, tridymite, and cristobalite

5 Galvanic currents produced by mixtures of electromotively dissimilar dusts produce active fibrogenesis

6 Fibrogenesis is accelerated when an animal prepared with a piezo electric dust is kept in a concentrated electromagnetic field of appropriate frequency

#### DISCUSSION

The present experiments were devised to determine whether substances possessing proper asymmetrical molecular space orientation and satisfying the other postulated properties for fibrogenic activity would behave in the manner expected. The results have clearly supported the concept and indicate that the phenomenon of piezoelectricity does exert biologic influence. Piezoelectricity has been defined earlier but it should be added here that, in addition to the direct and converse piezoelectric effects there are many related secondary effects. More study is needed to establish the forms and magnitudes of the energy sources occurring in nature and their biologic influence when converted by piezoelectricity. The piezoelectric property allows a theoretical transference of energy states in any direction between mechanical, electric, and thermal states.

There is a paucity of biologic and physical data from which one might define the physical behavior of minute solid deposits within tissue. On the other hand, the theory presented explains many previously unreconcilable observations and, in some instances is actually strengthened by these observations. The "chemical solution" theory of silicosis has presented many unsolved objections in addition to the important fact that fibrous tissue presents a poor barrier to substances in solution. The degree of solubility is not correlated with pathogenicity. The influence of the size of the particles has too sharp an end point to be a function of surface area alone. A critical number of particles is necessary to produce progressive fibrosis. A period of time is often observed between the termination of exposure and the onset of fibrosis, and, in clinical silicosis this interval often amounts to years. There has been no demonstration of either dissolution of crystals or actual solution in the areas of local involvement beyond that level of solution present in remote and uninvolved areas of the organism. All of these observations are irreconcilable with the solubility theory yet are either consistent with or strengthening to the theory being discussed. This present theory embodies the concept that naturally occurring sources of force are present in discon-

tinuous "bursts" of proper form and magnitude to effect intermittent piezoelectric responses in a proper "receptor," which responses constitute the fibrogenic stimulant

Many problems present themselves for future clarification. The known fibrogenic activity of several symmetrical crystals (talc, asbestos, etc.) is troublesome. This problem is presently under investigation in the light of "unstable crystal symmetry" and will be reported in more detail later. King, Gilchrist, and Rae<sup>11</sup> provided a clue when they reported that several benign shales and sericites became biologically active when treated with HCl. Guided by this observation, several samples of talc, asbestos, and mica have been similarly treated with chemically pure HCl and quantitative chemical determinations were made. As much as 4 per cent of the sample weight is lost, and this weight is recoverable in the HCl in the form of the chlorides of Al, Mg, Fe, Ca, Na, and other metals. When the residual material was subjected to x-ray diffraction, the patterns showed qualitative and quantitative changes by comparison with the original substance patterns. Further study in this regard with animal experiments will be forthcoming, but a statement is made at this point to emphasize that the material used in preparation of an animal may change to a pathogenic form after introduction into the animal.

Another problem requiring clarification is the observation that amorphous opal and our amorphous aluminum phosphate are both fibrogenic. This may be explained by the known tendency of amorphous material to crystallize when conditions are right. Amorphous aluminum is known to become crystalline in a quartz class when heated for purposes of sterilization.<sup>12</sup> The crystal state represents the preferred low energy state, and the amorphous state is maintained by a viscosity which precludes molecular reorientation. If the physical environment is proper, the amorphous material may return to the preferred crystal state. Diatomaceous earth is biologically inactive, but, after calcining at high temperatures, a diffraction pattern of cristobalite arrangement appears and the "new" dust develops a pathogenicity in keeping with its new crystal structure.<sup>13</sup> A similar perversion might occur in the animal tissue. This matter is under study with the hope that materials recovered from the fibrotic reaction sites may be subjected to x-ray diffraction for crystal orientation. A second possible explanation for the activity of amorphous materials is that piezoelectric properties may operate biologically at a molecular or unit cell level. This interpretation is not consistent with our experiences using ground fused (amorphous) quartz, which was benign by test. A third explanation is that electrical orientation might occur without true crystallization, and "electrets" with piezoelectric behavior might result. This has been accomplished when asymmetrical molecules are made to become electrically oriented without developing true crystal structure.<sup>14</sup> This consideration is presently under experimental test.

It also has been observed that one of the dusts (chalcopyrite), classified as benign in the studies of others,<sup>15</sup> belonged to an asymmetrical crystal class. Further consideration revealed that this dust was electrically conductive and

inertive piezoelectrically by test.<sup>7</sup> This exceptional instance stimulates speculation as to a functional explanation of the effect of aluminum therapy and of non hydroxide coating in modifying the fibrotic reaction to quartz. Polarity and, hence, piezoelectric activity might be "short circuited" by coating the particle with a thin skin of material of higher electric conductivity than the crystal itself or the tissue fluid around the crystal.

The piezoelectric mechanism requires an energy source, and several likely sources have been presented. Many observations have suggested that there are environmental factors influencing the incidence and character of disease states which cannot be explained in conventional terms. It is possible that environmental fluids of electromagnetic force can be of pathologic importance through the piezoelectric mechanism. If this be true a description of a biologic environment will remain incomplete until it includes a statement covering the frequency and intensity of force fields surrounding the host. This subject will be considered in more detail in subsequent studies.

Fibrous tissue makes its contribution to biologic economy by supplying physically supportive elements. When fibrous replacement represents the preponderant reaction to an injury one should suspect that injury to be of a physical order because it is this order of stimulation that fibrous tissue is functionally qualified to resist.

Within biologic variation fibrous tissue reactions are more "specific" to the site and degree of involvement than to the chemical nature of the fibrogenic material. Quartz, the tuberculous phospholipid and cholesterol have been shown to exert a biologic influence which is both interchangeable and 'symbiotically' additive when equal distribution is effected in similar tissue sites. This observation alone strongly suggests a common mechanistic relationship between the substances.

A mechanism has been demonstrated which exerts potent mitogenic influence when it acts in tissues. It is therefore important that those physiologic and pathologic states which are characterized by rapid proliferation of cells be restudied in the light of this mechanism. It is impossible to forecast where the mechanism will prove important. There are several biologic phenomena which seem specially suggestive. A classification of the problems under consideration and the basis for selection is presented. This classification is purely speculative and no commitments beyond the conclusions of this paper are intended. The classification is presented for the sole purpose of stimulating thought and of inviting collaboration and expansion.

### *Classification of Topics Suggesting Piezoelectric Mechanisms —*

#### *A Physiology*

- 1 Normal growth and differentiation (growth stimulating sterols)
- 2 Hormonal reactions as lactation and menstruation (sterols in general have very asymmetrical molecules)

#### *B Pathologic States*

- 1 Pathologic states at the site of strong and intermittent mechanical stress vectors
  - (a) Heart and major vessels (Aschoff bodies atherosclerosis)

- (b) Joints (Rheumatoid arthritis and allied states)
- (c) Thorax with cough and respiration (Pulmonary fibrosis in tuberculosis, silicosis, etc)
- 2 Situations where minute manifestations of potential could occur
  - (a) Hypersensitivity If the attractive charges of the antigen and the antibody were not dissipated at the point of contact when the immune body is formed, a minute dipole similar to that produced in the bimetal experiment would result (Overlaps with 1(a) for Aschoff bodies and with 1(b) in rheumatoid arthritis)
- 3 Situation in which there is an increase in metabolic products of asymmetrical molecular structure This includes both host and bacterial metabolic products
  - (a) Hypercholesterol and lipemic states (Overlaps with 1(a) in atherosclerosis and vascular disease)
  - (b) Tuberculous phospholipid (Overlaps with 1(c) in fibroid tuberculosis)
  - (c) Malignant change where associated with disturbed sterol metabolism and with large dose hormone therapy (Cancer of breast, prostate, testes, ovaries, and perhaps other neoplasms as well)
- 4 Pathologic lesions which conform architecturally with the equations of force such as Boeck's sarcoid, the other granulomata

It is again emphasized that this outline is for experimental organization and is not to be construed in any other light. It will be noted that all the classes outlined share in common the feature of cellular proliferation. In addition to cellular proliferation, each class possesses one or more of the mechanistic essentials of a piezoelectric reaction: (1) An acceptable energy source—heading B, 1. (2) An acceptable molecular receptor in excessive concentration—headings A, 1, A, 2, B, 2, B, 3 (a), B, 3 (b), B, 3 (c). (3) A manifestation of electrical potential—heading B, 2 (a).

B, 2 (a) classification is particularly speculative. This class is considered worth study as presented because of some recent highly significant observations. When a suspension of very finely divided biologically active dust is injected into the heart of a guinea pig, a "quick death" within a matter of minutes results. The death mechanism is clinically and pathologically indistinguishable from the "protein" death in guinea pigs that have been sensitized and shocked anaphylactically. This death reaction does not occur when benign dusts are similarly injected or when aluminum-hydroxide is added to the active dust suspension. We are indebted to Dr. Arthur Voisard for informing us of his remarkable experiment. It is felt that if an immune body failed to lose the attractive opposite charges of its antigen and antibody components due to abnormal electrical resistance at the area of contact, this immune body would become a minute dipole similar to the bimetal dipole discussed earlier and similar to the electric polarizations which appear on the surface of piezoelectric crystals. Again, the dampening effect of the aluminum hydroxide might be explained by the formation of a thin electrically neutralizing skin over the surface of the dust.

#### CONCLUSIONS

1 The ability of foreign materials to produce fibrogenesis is correlated with their crystalline structure.

2 Those tested amorphous materials and materials whose crystalline state possesses a central point of molecular symmetry (symmetrical crystals) are nonfibrogenic

3 Those tested crystals which do not possess a central point of symmetry (asymmetrical crystals) and possess piezoelectric properties are fibrogenic

4 Several materials previously untested biologically, selected for their physical properties, were tested and shown to be fibrogenic

5 Fibrous tissue reactions are considered to be stimulated by releases of energy in mechanical or electrical states within tissue

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## TISSUE RESPONSES TO PHYSICAL FORCES

### III THE ABILITY OF GALVANIC CURRENT FLOW TO STIMULATE FIBROGENESIS

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#### INTRODUCTION

A CONCEPT has been presented in earlier reports<sup>1,2</sup> which depicts fibrous tissue reactions as stimulated by and protective against releases of physical force. These earlier reports discuss a *modus operandi* whereby energy is transformed from a biologically noninfluential to a biologically active state. It was stated that separate reports would deal with the fibrogenic potential of individual energy states. As piezoelectricity is a property of certain materials to transform energy in any direction between motion, heat, and electricity, each of these energy states requires separate consideration.<sup>1,2</sup> When a piezoelectric crystal, e.g., quartz, which is deposited in tissues becomes squeezed by cough, strain, muscular activity, arterial pulsation, or any other physiologic means, it is capable of producing a current of electricity. The present report deals with the effect of such currents in tissue.

When two metals with dissimilar electromotive levels are connected by an electrolyte, they produce a flow of direct current. The characteristics of such a current differ from the current characteristics of a stressed piezoelectric crystal in the following manners:

#### Bimetal Battery Current

1. Current flow in one direction
2. Uninterrupted current flow
3. Limited duration—current will stop when the battery is "run down."

#### Piezoelectric Crystal Currents

1. Current flow in one direction while the crystal is stressed and in opposite direction when the stress is relieved
2. Interrupted current flow occurring only during stress and relief of stress
3. Unlimited duration as the crystal does not liberate energy but transforms energy from a source outside itself

In spite of these differences in current characteristics, there is sufficient similarity to make experimental consideration important.

In a bimetal electromotive battery, the voltage produced depends on the degree of electromotive dissimilarity of the metals and the resistance of the electrolyte (Ohm's law). As a similar technique is employed in similar animals, the factor of electrolyte resistance may be disregarded as a constant.

The importance of selecting identical metals which are electromotively neutral with tissues for screws and plates in orthopedic surgery has been emphasized by others.<sup>3</sup> It also has been shown that single metals may interact with the tissues in which they are implanted, resulting in current flow. The

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degree of fibrous tissue reaction has been shown to be proportionate to the amount of current produced.<sup>1</sup> These principles appear to be well established with large implants such as plates and screws. Heretofore, no data have been available describing the behavior of dust size bimetal deposits in tissues.

Silver, aluminum, and iron powders were selected for study. Aluminum is strongly positive electrically to silver and moderately positive to iron. Silver is strongly negative to aluminum and moderately negative to iron. Iron occupies a mid position electrically and is near the electromotive position of tissue fluids. The experimental procedure employed all three metallic dusts individually, as well as the three combinations of two mixed dusts. It is theoretically consistent to state that different levels of voltages were experimentally produced in tissue. The purpose of the experiment was to determine if the magnitude of the fibrotic response was proportionate to the voltage produced.

#### Theretical Voltage Table

Mixed Ag and Al	Highest voltage
Ag alone Al alone	Intermediate voltage
Ag and Fe Al and Fe	
Fe alone	Minimal or no voltage

#### MATERIALS

- Dusts 1 Aluminum metal dust  
2 Precipitated silver dust  
3 Iron powder

Animals Thirty four adult white rats were used in the experiment

#### METHOD

*Intraperitoneal Preparations*—One hundred milligrams of metallic dust were used in each experiment. This amount was either entirely of one dust, or, where two dusts were mixed, 50 mg of each fraction were used. All dusts were mixed immediately before injecting intraperitoneally into an anesthetized rat. The abdomen of the rat was then massaged to distribute the dust.

*Intratracheal Preparations*—The trachea of the anesthetized rat was exposed and the suspension of dust was injected into the trachea. Ten milligrams of a single dust or 5 mg each of mixed dusts were injected in each instance.

*Aluminum*—Three rats were prepared by simultaneous intraperitoneal and intratracheal injection. These rats were sacrificed at two, four, and six weeks.

*Iron*—Six rats were prepared intraperitoneally but not intratracheally. These animals were sacrificed as follows: one at four weeks, one at eight weeks, two at twelve weeks, and two at sixteen weeks.

*Silver*—Three rats were prepared intraperitoneally and intratracheally. They were sacrificed at one, three, and five weeks.

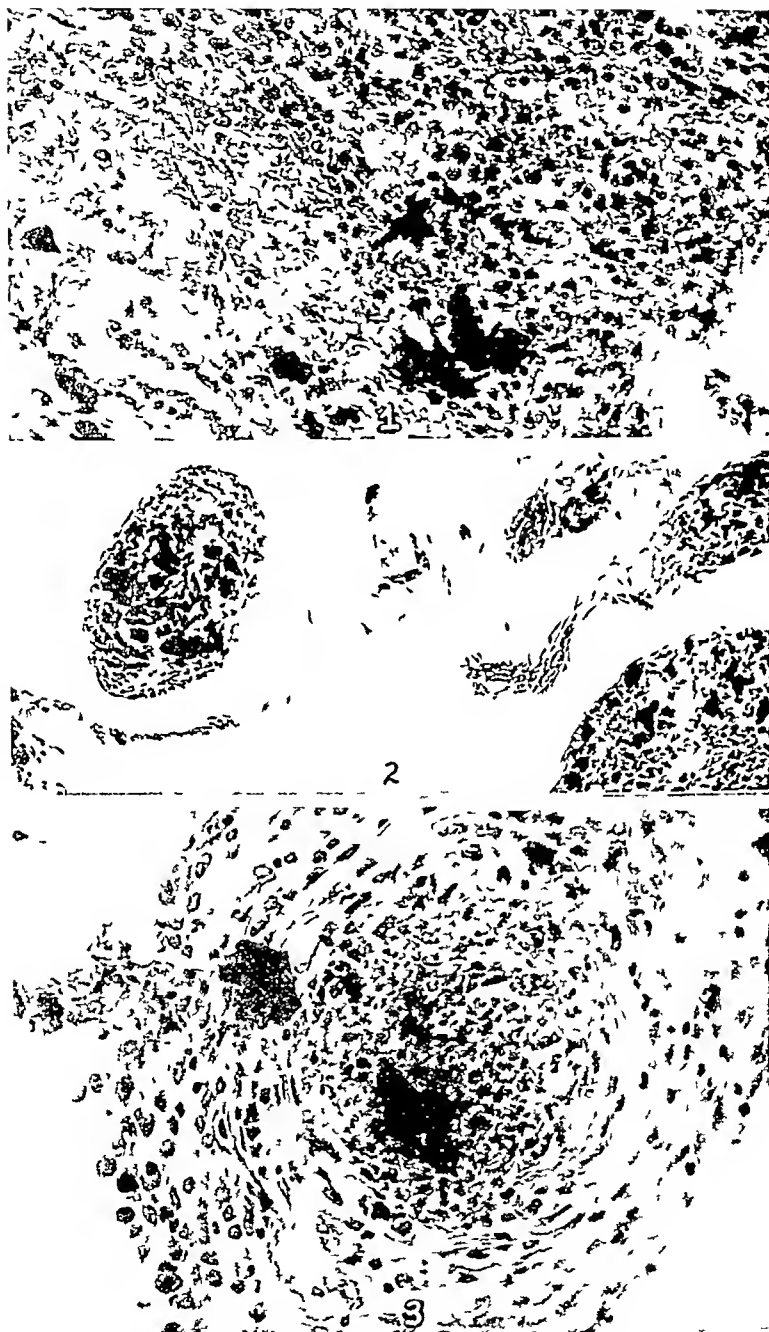
*Silver and Aluminum*—Six rats were prepared intratracheally and intraperitoneally. These animals were sacrificed at one, two, three, four, five, and six weeks.

*Silver and Iron*—Seven rats were prepared intraperitoneally but not intratracheally. These were sacrificed as follows: two at three weeks, two at four weeks, one at five weeks, and two at eight weeks.

*Aluminum and Iron*—Nine rats were prepared intraperitoneally but not intratracheally. These were sacrificed as follows: one at two weeks, two at four weeks, two at six weeks, two at eight weeks, and two at twelve weeks.

#### RESULTS

The results are described under four headings as determined by the magnitude of the reactions observed.



Reactions in the white rat (*Mus norvegicus*) to the highest theoretical voltage mixed silver and aluminum dust preparations

Fig 1—Reaction in the lung produced in two weeks ( $\times 400$ )

Fig 2—Intraperitoneal nodule produced in six weeks ( $\times 200$ )

Fig 3—Intraperitoneal nodule produced in two weeks ( $\times 400$ )



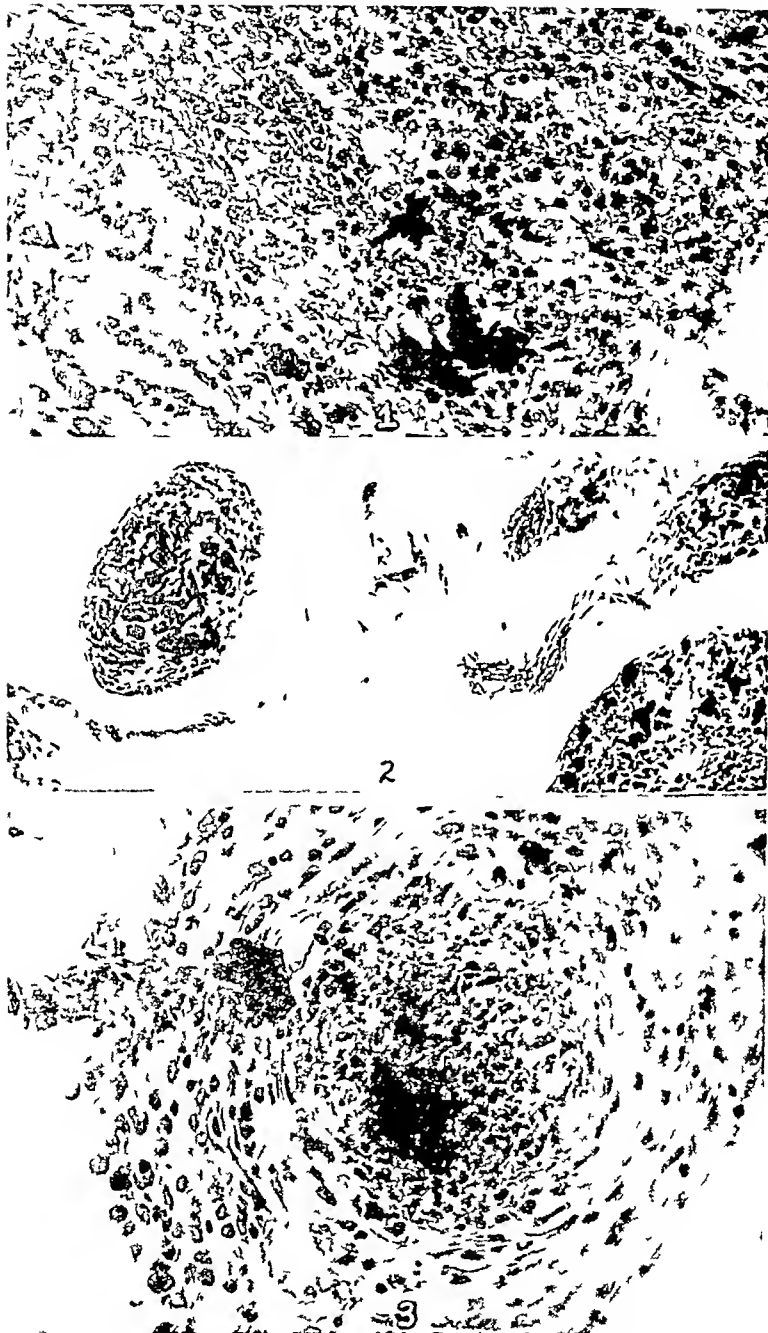


Reactions in the white rat to the second highest theoretical voltage, aluminum dust only

Fig 4—Intraperitoneal nodule produced in six weeks ( $\times 200$ )

Fig 5—Nodule on the surface of the liver produced in six and one half weeks ( $\times 100$ )

Fig 6—Nodules produced in the lung in six and one half weeks ( $\times 100$ )



Reactions in the white rat (*Mus norvegicus*) to the highest theoretical voltage, mixed silver and aluminum dust preparations

Fig 1—Reaction in the lung produced in two weeks (×400)

Fig 2—Intraperitoneal nodule produced in six weeks (×200)

Fig 3—Intraperitoneal nodule produced in two weeks (×400)

*II Silver Alone, Aluminum Alone*—The reactions to these dusts were similar histologically to the reactions to the mixed aluminum and silver preparations. The reactions were not as grossly extensive. Dense, collagenous, avascular, fibrous whorls were slower in formation. The lesions produced were considered as qualitatively similar to those produced by mixed aluminum and silver, but quantitatively less excessive and slower in development. See Figs 4, 5, and 6.

*III Silver and Iron, Aluminum and Iron*—These preparations produced a reaction with a less notable proliferation of fibroblasts and with minimal deposition of collagen. There was more vascularity and less extensive gross reaction than was seen in the preceding groups. Of particular interest was the microscopic appearance of a 'run down' electromotive battery. The two metals could be distinguished microscopically, and one metal was observed to "plate" the other.

*IV Iron Alone*—In this group the reactions were observed to be no more proliferative than is seen in simple foreign encapsulation.

#### DISCUSSION

The results obtained justify the conclusion that the magnitude of the reactions produced by electromotive emittents is proportionate to the voltage theoretically developed. Work is in progress to ascertain if the dielectric properties of mature fibrous tissue as compared with other tissues of the body, qualify fibrous tissue as relatively insulative and hence protective against electrical stimuli.

In these experiments aluminum or silver alone appeared more reactive than aluminum and iron or silver and iron. This is misleading. Iron is relatively near to animal tissue electromotively. The iron, therefore contributed nothing to the reactions which the surrounding tissue alone would not have supplied. It should be noted that, in the unmixed aluminum and the unmixed silver preparations, 100 mg were used intraperitoneally whereas in the aluminum and iron, and silver and iron mixed experiments only 50 mg of aluminum or silver were used. The factor of dosage therefore is obviously uncontrolled in these two groups.

The significance of these observations in the general problem of fibrotic reactions to disease has been discussed in earlier papers.<sup>1</sup>

#### CONCLUSIONS

- 1 Currents of electricity produced electromotively in tissues are capable of stimulating fibrotic reactions.
- 2 The magnitude of the reaction is proportionate to the voltage generated.

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# QUANTITATIVE SPECTROGRAPHIC ANALYSIS OF BLOOD AND TISSUE FLUIDS

ROBERT M. STECHER, M.D., HOWARD M. BEDELL, B.S., AND IRENE LEVIS, PH.D.  
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THE present study is limited to the quantitative determination of metallic elements in biologic material by the emission spectriograph using electrical excitation. Electrical excitation emission spectroscopy is to be distinguished from emission flame spectroscopy and absorption spectroscopy. Emission flame spectroscopy is limited in general to analysis of those elements having low excitation potentials such as the alkali metals. In flame spectroscopy the elements are excited to emit light by introducing the sample into a gas flame where the temperature is in the range of  $1,500^{\circ}$  to  $3,000^{\circ}$  C. By using electrical excitation where the sample is burned in an electric arc, temperatures of  $5,000^{\circ}$  to  $8,000^{\circ}$  C are obtained. It is possible, therefore, to excite even those elements having very high excitation potentials. Absorption spectroscopy lends itself best to the analysis of organic compounds in solution and is accomplished by passing light of all wave lengths through the sample and studying the absorption pattern obtained.

## THEORETICAL CONSIDERATIONS

Every element, when burned, produces radiations of monochromatic light which are specific. When such radiations are dispersed in a spectriograph and recorded on a photographic film they can be positively identified both qualitatively and quantitatively. The intensity of light emitted by any element during the burning is proportional to the amount of that element present. These are the principles upon which spectriographic analysis is based. In actual practice the amount of any element present in a sample is determined by measuring, with a microphotometer, the blackness of the photographic images of the specific spectral lines produced. A quantitative analysis of a sample for the metallic elements is made by comparing the spectrum of the sample with the spectrum of a known standard. The theoretical requirement of a standard is that it shall be identical with the sample in both physical structure and in general chemical composition. It is obvious that it is impossible to synthesize a real blood of known composition. The first necessity in the spectriographic analysis of blood becomes, therefore, that of converting the sample to a form which may be duplicated by a synthetic standard of known composition. This is best done by destroying the organic constituents by acid digestion or by low temperature dry ashing which also fixes the metallic elements as inorganic salts or oxides.

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The amount of any element present is determined by measuring the blackness of the photographic image produced. This is done with a micro photometer which determines by means of a photoelectric cell and galvanometer, the per cent of light transmitted through the line image. This transmittancy is inversely proportional to the intensity of light producing the image on the photographic plate according to Fig 1.

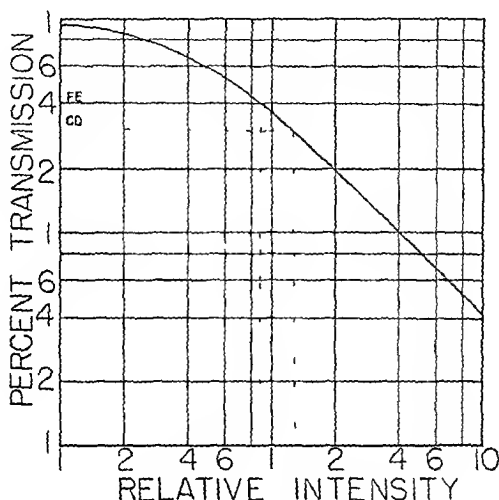


Fig 1—Characteristic curve of photographic emulsion

If the overall intensity of spectra could be reproduced accurately from sample to sample intensity of an element line could be plotted directly against the element concentration. This is not the case however so a correction for small variations in the intensity of spectra is made by use of the internal standard method which consists of adding a weighed amount of a foreign element to a measured amount of sample. The intensity of light emitted by the element used as internal standard is then accepted as a quantitative measure of the sample consumed or recorded. The element used for an internal standard must be chosen with care. It must be an element available in pure form, be chemically and spectrographically compatible with all elements to be assayed and have a simple spectrum which does not interfere with the sensitive lines of the trace elements.

The concentration of the unknown element is proportional to the ratio of the light emitted by that element to the light emitted by the internal standard. The ratio of light from the unknown element to light from the internal

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standard is called the intensity ratio. Actual computation of the results is done by applying the intensity ratio of the sample to the analytic working curves. Analytic working curves are established by burning a set of three or more synthetic standards containing the internal standard and known amounts of the elements to be analyzed in high medium, and low concentrations within the ranges expected. Such synthetic samples should be made to approximate the nominal composition for all elements of the material to be analyzed even though only one element is to be determined. Analytic

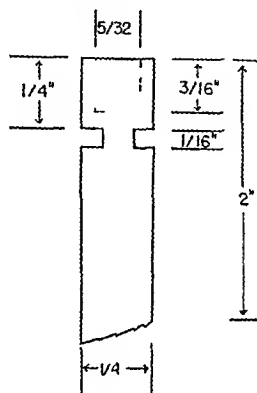


Fig 4—Shape of graphite electrode used

working curves have to be determined separately for each spectriograph and redetermined from time to time for the same instruments. The analytic working curves for lead (Pb), iron (Fe), magnesium (Mg) and sodium (Na) in blood used in these studies are shown in Fig 2, and for lead, magnesium, and phosphorus (P) in urine in Fig 3.

#### PROCEDURE

The first work and that from which the data and analytic curves in this paper were obtained was based on variations of a wet ashing technique, using a mixture of perchloric and nitric acids to destroy the organic material in the blood. The best results by the acid digestion method were obtained by digesting 5 cc of blood in 15 cc of  $\text{HClO}_4$  plus  $\text{HNO}_3$ , the acids being in the ratio of 2:1. The mixture of blood and acids was taken to dryness and the residue picked up in 5 cc of nitric acid solution containing 4 Gm of cadmium acetate per 100 cc of acid. Bismuth was used in place of cadmium as an internal standard with good success particularly in the determination of lead. Synthetic standards simulating the mineral content of blood are made by dissolving pure salts in a dilute nitric acid solution.

After digestion, 0.02 or 0.05 c.c. of sample, depending on sensitivity required, is then pipetted into the cup of a pure graphite electrode. The electrode used is of the type shown in Fig. 4. The sample is dried in the cup of the electrode by the heat of an infrared lamp. Best results are obtained by using electrodes in which a drop of pure liquid petrolatum has been allowed to soak in and air-dry before the sample or the standard is put into the cup. This prevents the sample solution from soaking deeply into the graphite and gives a deposit of salts in the cup on drying. The sample is then burned in a direct current arc with a current of 15 amperes. A burning time of about ten seconds is sufficient to completely volatilize the sample when the oil-treated cup is used. The synthetic standard is burned in juxtaposition on the same photographic film to facilitate quantitative determinations.

A microtechnique was developed using a sample of only 0.02 c.c. of blood obtained either by a skin puncture or from a sample of heparinized blood taken by venepuncture. The sample is measured in a standard Sahli hemocytometer and is delivered into the cup of a pure graphite electrode. The sample is covered with the same amount of concentrated perchloric acid to which 4 Gm. of cadmium acetate per 100 c.c. have been added as internal standard. Heat is applied gently to the electrode with an infrared lamp for about thirty minutes until the specimen is digested. The sample is burned in a direct current arc as before. Again a synthetic standard sample is burned and recorded in juxtaposition on the same photographic film. Some difficulty was encountered because of the rapid reaction of the blood with the perchloric acid and also because of the difference in the absorption of the blood and of the synthetic standard solutions into the graphite cups and the resulting different rates of volatilization into the arc. Reasonable duplication of results was obtained under carefully controlled conditions.

A dry ashing technique was developed and tried which gives excellent results with minimum difficulty. A small piece of ashless filter pad is packed into the cup of the electrode. Samples of both the blood and the synthetic standard are prepared in identical fashion by pipetting 0.02 c.c. into the pad, then 0.02 c.c. of cadmium acetate solution is added. The electrodes are placed in an oven controlled at 850° F. maximum. Ashing is complete in about one to one and one-half hours. An excessive ashing time may cause the loss of any of the more volatile elements. A small deposit of mineral ash is left in the bottom of each electrode and there should be no absorption of any of the minerals into the graphite of the electrodes. The deposit is rather light and fluffy, so that to prevent loss at the initiation of the arc a drop of  $\text{HNO}_3$  should be put in each cup. The sample is caked and some of the minerals are dissolved and carried into the surface of the graphite. A small amount of penetration is not objectionable provided that it is uniform with both the unknown and the standard solutions. The electrodes are then burned in the direct current arc as before.



Another treatment of the sample after ashing which shows good promise, particularly in stabilizing the arc and enhancing sensitivity, is that of packing lithium carbonate into the cup on the ash. The ash is fused with the lithium compound on heating in the arc and the burning characteristics are those of the lithium compound. The cadmium is still used as the internal standard and an exact measurement of the lithium is not required. This method is very similar to that reported by Myers<sup>1</sup> and others in the analysis of leaves and other plant tissue for both major and trace mineral elements.

After burning the samples in any of the described techniques, the film or plate is developed under rigidly controlled conditions as to temperature and time, and the measurements of the line intensities are made with the microphotometer. Manufacturers' specifications for film processing are followed.

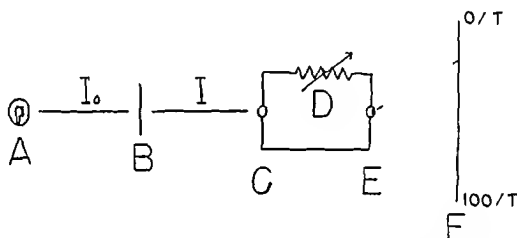


Fig 5—Schematic diagram of microphotometer. A Constant light source. B line image on film. C photocell and slit. D variable resistance. E galvanometer. F meter scale.  $I_0$  original light intensity.  $I$  transmitted light intensity.  $T$  transmission.

#### CALCULATIONS

The method by which the ratio of the intensities of two spectral lines is determined is as follows:

The transmittancy of the line image on the photographic plate is determined by means of the microphotometer as illustrated in Fig 5 which shows a schematic diagram of the microphotometer.

The galvanometer circuit is adjusted to read zero deflection when no light strikes the photocell and to read 100 when the light passes through clear film. Then the reading obtained when a line image is interposed between the light source and the photocell is called per cent transmission ( $T$ ). It is obvious that the blacker the line on the film, the lower the reading ( $T$ ) which will be obtained. This in turn indicates a stronger intensity of light emitted by the element under study which produced the image. The relationship of ( $T$ ) with respect to relative intensity ( $I$ ) of light emitted by the elementary sample is shown in Fig 1. If for example as shown in Fig 1, in the analysis of blood for iron the values for  $T$  are  $T_{Fe} = 40$  and  $T_{Cd} = 30$  then  $I_{Fe}/I_{Cd} = 0.92/1.28 = 0.72$ . From Fig 2 the concentration of iron would be found to be 27 mg/100 cc of whole blood.

TABLE I COMPARISON OF CHEMICAL AND SPECTROGRAPHIC ANALYSIS OF 25 FE DETERMINATIONS OF WHOLE BLOOD IN MG PER 100 CC

SPECTROGRAPHIC	CHEMICAL	DIFFERENCE
34	35.2	+1.2
34.2	37.8	+3.6
35	36.1	+1.1
37.1	34.0	-3.1
37.1	39.0	+1.9
37.5	36.9	-.6
38.2	41.9	+3.7
38.3	41.9	+3.6
38.3	38.0	-0.3
39.1	41.1	+2.0
40.5	40.1	-.4
43	45	+2.0
43	46.0	+3.0
43.6	45.8	+2.2
45.5	49.0	+3.5
46.0	49.0	+3.0
46.2	49.0	+2.8
46.2	51.9	+5.7
46.2	51.9	+5.7
47.9	51.0	+3.1
49.6	48.1	-1.5
50.5	48.3	-2.2
51.9	51.8	-.1
53.0	56.0	+3.0
55.0	55.0	+0.0

## RESULTS

Observations were first made on iron because it was easy to check spectriographic results by chemical methods. A series of determinations on twenty-five different samples of blood chosen at random from the medical service at City Hospital is shown in Table I. The results are arranged in ascending order of magnitude as determined by this spectriographic method. The values ranged from 34.0 to 55.0 mg per 100 cubic centimeters. The iron content was determined chemically. The results obtained by both methods were in essential agreement. The actual deviation of the spectriographic method from the chemical method ranged from +5.7 to -3.1 mg per 100 cubic centimeters. The average difference was +1.64. These deviations were from +12 per cent to -8 per cent, with an average of +5 per cent. The standard deviation for the series was 6.06 per cent of the amount present.

After showing that the iron determinations obtained spectriographically compared favorably with those obtained by conventional chemical tests, recovery and reproducibility studies were made. Reproducibility of iron is

TABLE II REPRODUCIBILITY DETERMINATIONS ON OX BLOOD WITH ADDED MINERALS (MG PER 100 CC)

	Fe	Na	Mg	Pb
Sample 1	42	320	3.9	9
	42	340	4.1	11
Sample 2	76	410	9.6	5
	80	385	8.6	5
	80	380	8.0	6

shown in Table II. Sample 1 is that of a sample in the low normal range of concentration, whereas Sample 2 is a normal blood to which none has been added. The non-determinations checked within 5 per cent in the three determinations on the second sample and were identical for the duplicate runs of Sample 1.

Observations on sodium are shown in Table II. Good reproducibility within 7 per cent of the amount present was obtained on both samples. Sample 2 demonstrates good recovery, because it is Sample 1 to which 50 mg of Na per 100 cc were added. Results for Pb shown in Table II and Table III demonstrate both recovery and reproducibility. All samples were normal blood to which calculated amount of Pb were added to give 0.05, 0.1, 0.2, 0.4, 0.5, and 1.0 mg per 100 cubic centimeters. Correction was made for the original Pb in the blood.

TABLE III THE RECOVERY OF LEAD ADDED TO WHOLE BLOOD (MG PER 100 CC)

Lead added	0.025	0.05	0.10	0.20	0.40
Lead recovered		0.047	0.095	0.185	0.36
		0.051	0.105	0.190	0.38
		0.065	0.150	0.16	0.7
Sample 1		0.049	0.095		
		0.062			
Sample 2	0.025	0.05	0.10	0.21	0.41
	0.023	0.045	0.10	0.212	0.43
					0.42
Sample 3		0.045	0.08	0.19	
		0.056	0.11	0.21	

Tests were equally satisfactory for magnesium.

Any of the body fluids which may be measured onto the filter pad in the electrode may be analyzed in exactly the same manner. Standards prepared to have mineral concentration simulating those normally present in the body fluid under study are required for accurate quantitative analysis.

#### EQUIPMENT

These studies were started on an original grating spectrograph having high resolving power and high dispersion. Such an elaborate instrument is not necessary because subsequent experience proved that equally accurate results were obtained with a less expensive instrument (Jurell Ash 3 meter replica grating Wadsworth mount). The smaller instrument because of its higher wave length coverage and increased speed, is more desirable for this work in some respects than the larger instrument. The small instrument covers in one exposure the entire spectral range necessary to determine all the elements from potassium at  $7800 \text{ \AA}^\circ$  in the high visible range to phosphorus at  $2500 \text{ \AA}^\circ$  in the low ultraviolet.

## SUMMARY

Emission spectriography offers a convenient, accurate, and rapid method of making simultaneous quantitative determinations for several elements in one sample. Although the method has so far been tested only for iron, sodium, potassium, magnesium, calcium, and lead, we are confident that it can be readily adapted to many more.

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# THE USE OF THE EMISSION SPECTROGRAPH FOR THE QUANTITATIVE DETERMINATION OF Na, K, Ca, Mg, AND Fe IN PLASMA AND URINE

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**E**MISSION spectrography is a common industrial procedure for the analysis of alloys. Each element is recognized by the characteristic spectrum emitted when it is subjected to high temperatures in an electric arc or spark. The spectrum is dispersed by means of a grating or prism which separates the lines to a fraction of an angstrom. The spectrum is photographed on calibrated plate or film.<sup>1, 2</sup> The density of the spectral lines is related to the concentration of the element. Quantitation is obtained by the incorporation of a known amount of a foreign element which serves as an internal standard.<sup>3</sup> By photoelectric comparison of the densities of the spectral lines of the element being determined and the internal standard the concentration may be obtained.

Numerous methods for the analysis of solids and liquids, using both arc and spark sources, have been described by Brode.<sup>4</sup> The customary, but time consuming, procedure for the analysis of solutions is to evaporate to dryness in a craterized carbon electrode before ignition. Solutions also have been analyzed by direct introduction through a capillary into the electric source,<sup>5</sup> but in our experience this method has been unsatisfactory. The special feature of the method reported in this communication is the employment of a rotating disk electrode, or rotode, one section of which is partially immersed in the prepared biologic fluid. As the disk rotates the sample is picked up on the surface of the electrode and brought under the influence of high voltage spark excitation.

## PREPARATION OF SAMPLE

The purpose of sample preparation is threefold: (1) destruction of interfering organic matter, (2) incorporation of a known amount of a foreign element as an internal standard, (3) adjustment of final volume to make spectral lines of optimal intensity.

Prior destruction of plasma and urinary protein is necessary to prevent coagulation during sparking and consequent irregularity in cation vaporization. Direct treatment of the plasma with warm tetraethyl ammonium hydroxide is the simplest method for destruction of protein and produces a sample suitable for spectrographic analysis of Na, Ca, Mg and Fe. However, the ammonium ion impairs sensitivity to such an extent that the intensity of the potassium lines is reduced below a point where they can be read with accuracy. A 2:1 nitric perchloric acid mixture is adequate for the destruction of protein and

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nouprotein nitrogenous compounds in the plasma and produces a sample suitable for the determination of K as well as Na, Ca, Mg, and Fe. To effectively destroy the large amounts of urea in urinary samples, it is necessary to use hydrochloric acid in addition to the nitric-perchloric mixture.

Lithium, cadmium, nickel, and copper were tried as internal standards and lithium was found to be most suitable. In the event that lithium is used clinically as a salt substitute, it will be necessary to change the internal standard. Either cesium or rubidium should be satisfactory.

*Plasma*—Samples must be collected in glass stoppered containers to prevent the adsorption of cations by cork or contamination by rubber stoppers. Four cubic centimeters of heparinized plasma are pipetted into a 250 ml beaker, to which are added 15 cc of 2:1 nitric-perchloric acid mixture\* and 3.0 cc of a 0.3 Gm per cent lithium chloride solution. The lithium serves as an internal standard. The beaker is covered with a watch glass and the solution is permitted to evaporate to incipient dryness. Five cubic centimeters of distilled water are added to the cooled beaker and the contents swirled until the solution is complete. This completes the preparation of the sample for spectriographic analysis.

*Urine*—To 10.0 cc of urine in a 250 ml beaker are added 10 cc of hydrochloric acid and 15 cc of 2:1 nitric-perchloric acid mixture and 3.0 cc 0.3 Gm per cent lithium chloride solution. The sample is treated in the same manner as for plasma, except that the final volume is made up to 10.0 cc with distilled water.

#### APPARATUS AND PROCEDURE

The apparatus in use is an Applied Research Laboratories 15 meter grating spectriograph equipped with an ARL rotode, as shown in Fig. 1. The rotode is driven by a synchronous motor supplied with a reduction system to control the speed of rotation. A high-purity carbon disk electrode  $\frac{1}{2}$  in in diameter and  $\frac{1}{8}$  in in width with a centered  $\frac{1}{8}$  in hole is attached to a carbon axle which is inserted in the rotating adapter of the electrode. This system forms the lower electrode of the spark stand (Fig. 1, A). The upper electrode is a  $\frac{1}{4}$  in tapered high-purity graphite rod (Fig. 1, B). A porcelain boat containing the prepared sample is arranged on an adjustable platform, which may be elevated to bring the solution into contact with the rotating disk (Fig. 1, C). A speed of four revolutions per minute was found optimal for the analyses carried out in this study.

The excitation source used to vaporize the samples is an Applied Research Laboratories high-voltage spark source unit. The input is set at 240 volts. The rotary spark gap is adjusted so that the meter recording relative primary voltages reads 75 volts. Power is set at 2 kv-a. Full inductance of this unit is employed (setting at 8). The electrodes are pre-sparked forty seconds before the disk is brought into contact with the solution. A second pre-spark is carried out after immersion of the rotating disk to concentrate the solutes on the carbon

\*Perchloric acid should never be brought into contact with organic material in the absence of nitric acid because of the danger of explosion. The addition of nitric acid permits smooth oxidation of organic material with safety.

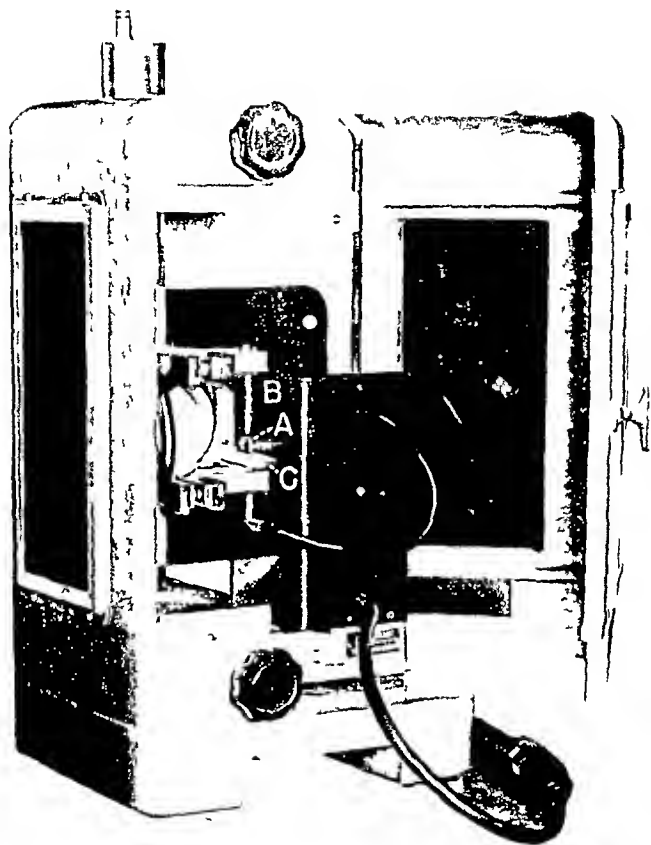


Fig 1—A Rotating disk electrode B Graphite rod electrode C Porcelain boat.

surface. Three separate exposures of forty seconds each are then made of a given sample. The light emitted by the vaporization of the solution is transmitted by means of a cylindrical lens through the slit of the spectrograph, dispersed by the grating and focused onto a 35 mm spectrum analysis #1 film. This film is developed using D 19 developer for two minutes at 70° F. A single film is sufficient for nine exposures.

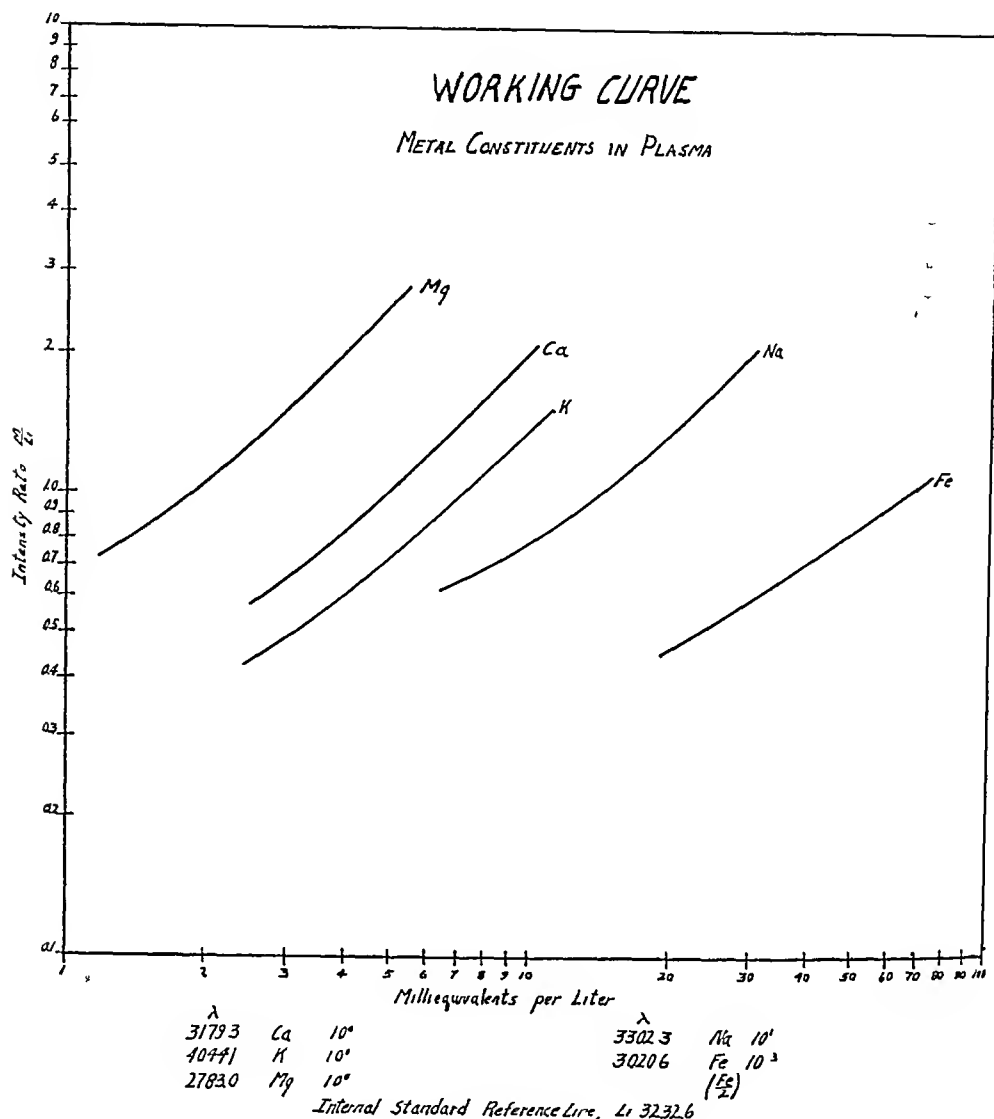


Fig 2

This procedure obtains the quantitative spectriographic analysis of sodium, potassium, calcium, magnesium, and iron. The wave lengths found most suitable for quantitation are sodium, 3302.3 Å, potassium, 4044.1 Å, calcium, 3179.3 Å, magnesium, 2783 Å, iron, 3020.6 Å, lithium reference line, 3232.6 angstroms. The films are read on an ARL densitometer. Samples are generally run in lots of twelve. Approximately two hours are required from the beginning of sample preparation until results are available for the five metals in each of the twelve samples.

Synthetic standards, approximating the electrolyte composition of plasma and urine, are run at least twice daily as a control on spectriographic accuracy.



These standards are subjected to the same preparation as described for plasma and urine samples. Working curves and rapid calculating scales<sup>7</sup> are established from these standards so as to include a range covering one half to two times the expected concentrations (Fig 2)

TABLE I RESULTS OF SEVEN SEPARATE EXPOSURES OF A SINGLE SAMPLE OF PLASMA

Na	K	Ca	Mg	Fe
131	5.5	5.2	2.7	0.038
128	5.5	4.8	2.5	0.038
128	5.5	5.0	2.5	0.036
130	5.0	5.0	2.5	0.036
130	5.0	5.0	2.4	0.036
135	5.0	5.2	2.5	0.034
130	5.1	5.6	2.4	0.036

Concentrations are expressed as milliequivalents of metal per liter of plasma

## RESULTS

The results of seven separate determinations on the same sample, picked at random, are given in Table I to illustrate spectrographic reproducibility and precision.

To determine the accuracy of recovery, known amounts of individual metallic salts were added to previously analyzed samples of plasma or to synthetic standards. Table II represents a portion of the results picked at random.

TABLE II COMPARISON OF AMOUNTS FOUND ON SPECTROGRAPHIC ANALYSIS WITH THOSE KNOWN TO BE PRESENT IN A SERIES OF FORTIFIED SAMPLES

AMOUNT PRESENT	AMOUNT FOUND	PER CENT ERROR
Na	Na	
195	177	- 9.2
105	185	+ 5.1
260	252	- 3.1
200	250	+ 16
322	336	+ 4.4
208	215	+ 3.5
K	K	
7.6	7.3	- 4.0
7.0	7.7	+ 13
7.0	7.4	+ 20
7.6	7.0	- 9.2
7.6	7.8	+ 2.0
10.2	10.2	± 0.0
Mg	Mg	
2.50	2.36	+ 2.4
3.32	3.24	- 2.4
4.16	3.74	-10.0
Ca	Ca	
6.2	6.2	± 0.0
6.2	6.4	+ 3.2
6.2	6.6	+ 6.4
7.4	7.8	+ 5.4
7.4	7.8	+ 5.4
7.4	7.0	- 5.4
7.4	7.6	+ 2.7
Fe	Fe	
0.07	0.068	- 5.5
0.108	0.106	- 1.8
0.072	0.078	+ 8.3
0.108	0.122	+13.0
0.108	0.108	± 0.0

Concentrations are expressed as milliequivalents of metal per liter of plasma.

to illustrate the percentage error of the spectrographic determinations. The error is usually within the range of plus or minus 5 per cent and almost always within plus or minus 10 per cent, regardless of the amount present in the samples. Determinations of Na, K, Ca, Mg, and Fe in the plasma of one hundred normal subjects are in progress and will be reported in a separate communication.

#### SUMMARY

A rapid and accurate method employing the emission spectrograph and featuring a rotating disk electrode has been described for the quantitative analysis of Na, K, Ca, Mg, and iron in plasma and urine.

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## ANTITHROMBIN AND HEPARIN IN HUMAN BLOOD

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HOWELL<sup>1, 2</sup> originally postulated the presence of a factor or its precursor in plasma which influences the ant clotting effect of heparin. The precursor he referred to as proantithrombin and the activated factor as antithrombin. It was believed that one of the functions of heparin is to promote the production of antithrombin from proantithrombin.

Heparin alone has little if any antithrombin activity when tested with purified fibrinogen and thrombin solutions. However, it is uniformly recognized that heparin appears to react with a substance present in normal plasma, leading to the formation of a substance which is capable of inactivating thrombin. Excellent reviews of the literature on heparin by Mason<sup>3</sup> and Eagle<sup>4</sup> discuss these factors. Quick<sup>5</sup> reported that a normal antithrombic substance exists in the albumin fraction of plasma. He stated that heparin merely intensifies the existing antithrombic property of the albumin fraction. Astup and Darling<sup>6, 7</sup> have shown that slight amounts of an antithrombic substance exist normally in ox plasma and that this substance is not identical with the factor which is activated by heparin.

This report is concerned with the further identification of the factor in human plasma which in the presence of heparin results in the formation of an active antithrombic substance.

### EXPERIMENTAL PROCEDURE AND RESULTS

All early experiments were conducted on reconstituted dried normal human plasma (obtained from volunteers by the American Red Cross) or fractions obtained from this plasma by ammonium sulfate fractionation. Later experiments were conducted using human blood fractions obtained by the low salt low temperature alcohol fractionation method.<sup>8</sup> The experiments were conducted by mixing the various substances in serologic glass tubes which were supported in a constant temperature water bath maintained at a temperature of 38° C. In all experiments the various agents were added to the tubes in the bath in as rapid succession as possible from automatic pipettes, always adding the thrombin last. When the thrombin had been added the contents of the tubes were thoroughly mixed by vigorously swirling the tube in the air immediately above the bath. Clotting times were noted by an interval timer. The tubes were tipped every ten seconds or as often as necessary to determine the earliest formation of a definite gelatinous clot which could be observed grossly when the tube was tipped. Clotting times thus represent the time interval lapsing between the addition of thrombin and the formation of the clot.

An optimal concentration of heparin for use in the subsequent experiments was determined which would increase slightly the clotting time of plasma when added to a mixture of thrombin and plasma. The effect of varying the amount of thrombin was then determined. The results are shown in Fig. 1. The figure demonstrates that the ability of heparin

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to prevent the clot formation is inversely related to the concentration of thrombin. The antithrombin activity resulting following the addition of heparin to plasma becomes markedly more evident as the thrombin concentration is diminished

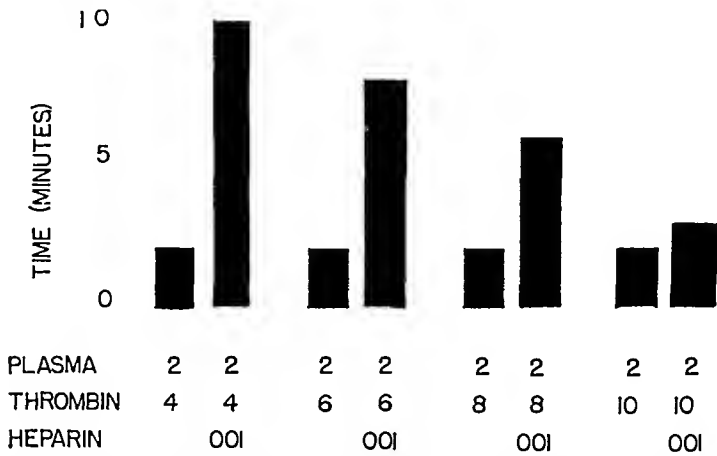


Fig 1—The effect of heparin on the clotting time of reconstituted human plasma as produced by the addition of varying amounts of thrombin. Quantities of plasma as indicated are in milliliters thrombin (Thrombin Topical Parke-Davis) is in units and heparin (Lilly) is in milligrams. All volumes were made constant (10 ml) by the addition of citrate-phosphate buffer (MacIlvane's standard buffer solutions) of pH 7.3

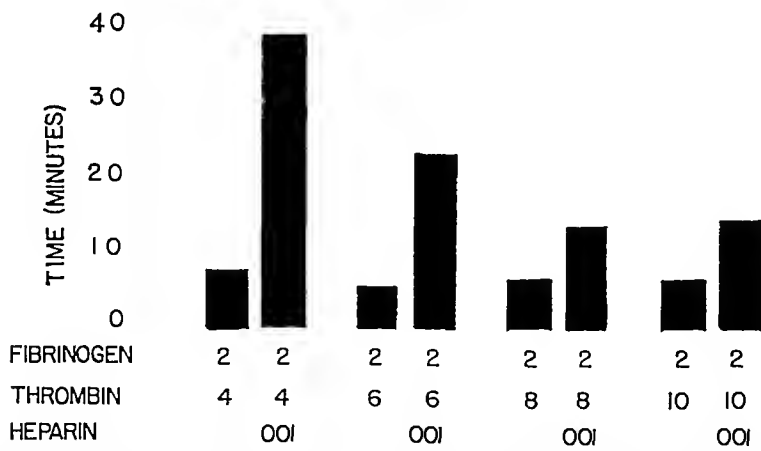


Fig 2—The effect of heparin on the clotting time of partially purified human fibrinogen as produced by the addition of varying amounts of thrombin. Quantities of fibrinogen solution as indicated are in milliliters of 0.4 per cent solution of fibrinogen thrombin (Thrombin Topical Parke-Davis) is in units and heparin (Lilly) is in milligrams. All volumes were made constant (10 ml) by the addition of citrate-phosphate buffer of pH 7.3

The foregoing series of experiments was then repeated except that partially purified fibrinogen was substituted for the plasma. The fibrinogen was made in this laboratory by ammonium sulfate fractionation\*. The results are shown

\*This fibrinogen preparation was made by precipitation from reconstituted normal human plasma using 20 per cent saturation with ammonium sulfate. Removal of the precipitate by centrifugation and redissolving it in water and reprecipitating it two times with 20 per cent saturation of ammonium sulfate. The final precipitate was dialyzed using heavy-weight vegetable parchment casings in cold running tap water until the dialysate was free of sulfate. The dialyzed preparation was centrifuged and the precipitate was removed and dried in a desiccator divided into portions and stored at 0° C until used. When the preparation was used it was dissolved in 0.1N citrate phosphate buffer (MacIlvane's standard buffer solutions) of pH 7.3

in Fig 2 This figure shows that the rapidity of the conversion of the partially purified fibrinogen to fibrin in the presence of heparin and thrombin was inversely related to the amount of thrombin added Furthermore the clotting time of fibrinogen with thrombin in the absence of heparin was not influenced by the amount of added thrombin over the range studied The antithrombin activity which resulted following the addition of heparin in the presence of partially purified fibrinogen becomes more evident as the concentration of thrombin is diminished

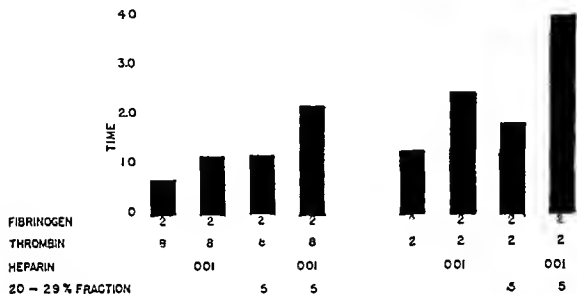


Fig 3—The effect of heparin in the presence of plasmin (20-29% fraction) on the clotting time of partially purified human fibrinogen as produced by the addition of varying amounts of thrombin. Quantities of fibrinogen as indicated are in milliliters of 0.4 per cent solution of fibrinogen; thrombin (Thrombin Topical Parke-Davis) is in units; heparin is in milligrams and the 20-29% ammonium sulfate fraction is in milliliters of 1.0 per cent solution of the fraction. Time is in minutes. All volumes were made constant (1.0 ml.) by the addition of citrate-phosphate buffer of pH 7.3

The next series of experiments involved the determination of the presence of a precursor of an antithrombin substance in the euglobulin fraction of normal human plasma. The euglobulin fraction (fraction obtained in the 20 to 29 per cent saturation range with ammonium sulfate) used in the experiments was made by the procedure outlined by Loomis, Charles, and Rider<sup>9</sup> which they used for the purification of fibrinolysin (plasmin)<sup>10</sup>. This fraction will be referred to in this paper as the plasmin fraction obtained by ammonium sulfate fractionation. Fig 3 illustrates the results when this fraction is added to the fibrinogen-thrombin-heparin mixtures. The figure illustrates that although the plasmin fraction has very little antithrombin property in itself, the clotting time is greatly prolonged in the presence of plasmin and heparin. In a similar type of experiment it was found that neither the 29 to 50 per cent nor the 50 to 75 per cent (albumin) fractions contained an antithrombin substance and they were not capable of being activated by heparin.

The next series of experiments involved the use of plasma fractions which were obtained by the low salt, low temperature alcohol fractionation technique. Fraction I was used as the source of fibrinogen. Fraction III-2 was used as the source of thrombin. Fraction III-3 (plasmin fraction) was tested for anti-

thrombin activity No antithrombin activity was found in this fraction Fraction III-3 was found to contain some traces of thrombin as evidenced by its ability, in high concentrations, to cause the clotting of fibrinogen

Fig 4 shows the results obtained using alcohol fractions The amounts of Fraction III-3 used in the experiments shown in Fig 4 did not contain a sufficient amount of thrombin to convert fibrinogen to fibrin The figure shows

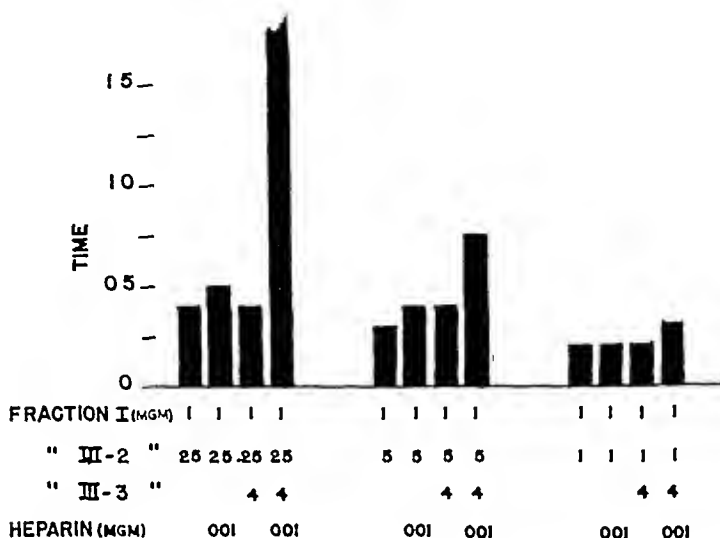


Fig 4—The effect of heparin in the presence of Fraction III-3 (plasmin) on the clotting time of Fraction I (fibrinogen) as produced by the addition of varying amounts of Fraction III-2 (thrombin). Time is in minutes. All quantities are in milligrams and all volumes were made constant (1.0 ml.) by the addition of citrate-phosphate buffer of pH 7.3

that although neither heparin nor Fraction III-3 had a definite marked antithrombin action in themselves, when the two were added to a fibrinogen-thrombin mixture a definite marked increase in the clotting time resulted. As the concentration of thrombin was decreased, the antithrombin action of the mixture of Fraction III-3 and heparin became more apparent.

Further experiments demonstrated clearly that the precursor of the antithrombin was in Fraction III-3 and was inactivated after exposure to 70° for as short a time as one minute. At 60° C the destruction of the antithrombin precursor was less rapid but occurred after five minutes of exposure. It is stable at 40° for four hours. In contrast to this, the exposure of heparin to temperatures as high as 90° C for five minutes did not influence its ability to activate the precursor of antithrombin in Fraction III-3.

#### DISCUSSION

Howell<sup>1, 2</sup> believed that heparin prevented the clotting of blood by a dual action. These actions are (1) stabilizing prothrombin through combination with it and (2) promoting the production of antithrombin from its precursor, proantithrombin. Mellanby<sup>11</sup> believed that heparin does not act as an antiprothrombin but that its exclusive action is that of being capable of producing an

antithrombic substance in blood. Quick believed that heparin acts by activation of an antithrombin precursor which he stated was in the albumin fraction. The foregoing experiments did not show evidence of a normal antithrombin activity of the albumin fraction of reconstituted human blood. Furthermore, there was no evidence of significant amounts of antithrombin action of the plasmin fraction whether the fraction was made by ammonium sulfate or alcohol precipitation techniques. The fraction of human blood designated as the plasmin fraction (by ammonium sulfate fractionation) and Fraction III 3 obtained by the alcohol fractionation method showed muted antithrombin activity only in the presence of heparin.

In the experiments reported here when heparin is added to reconstituted normal human plasma or to partially purified fibrinogen in the presence of thrombin the clotting time was found to be inversely related to the concentration of thrombin. In such a test heparin appears to have a definite antithrombin activity. When heparin is added to Fraction I (fibrinogen) in the presence of Fraction III 2 (thrombin) obtained by low salt low temperature alcohol fractionation the clotting time is only slightly prolonged. In such a test no definite pronounced antithrombin activity for heparin could be demonstrated. This confirms previous work<sup>1, 2, 6</sup> which stated that heparin has no antithrombic action when tested on purified fibrinogen and thrombin.

The plasmin fraction obtained by ammonium sulfate fractionation and the plasmin fraction (Fraction III 3) obtained by alcohol fractionation were found to be practically free of inherent antithrombin activity. When heparin was added to either of these fractions in the presence of fibrinogen and thrombin the clotting time was greatly lengthened. The clotting time in such a test as that described is inversely related to the amount of added thrombin, therefore a true antithrombic substance is formed. In the presence of heparin the true antithrombin is formed from a precursor which is present in the plasmin fraction or Fraction III 3 and which has the characteristics of Howell's proantithrombin. It is inactivated rapidly by exposure to a temperature of 70° C.

#### SUMMARY

The effect of heparin on the fibrinogen-thrombin reaction in human blood is that of promoting the liberation of antithrombin from its precursor proantithrombin. Proantithrombin is present in the plasmin fraction obtained by ammonium sulfate fractionation and in the plasmin fraction (Fraction III 3) obtained by alcohol fractionation.

The author wishes to express his appreciation to Dr. F. F. Johnson of the Cutter Laboratories, Berkeley, Calif. for generously supplying the plasma samples obtained by alcohol fractionation methods. The technical assistance of Miss Evelyn Adams is gratefully appreciated.

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## BONE MARROW STUDIES IN THE POLYCYTHEMIA OF HIGH ALTITUDES

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IT IS generally accepted that the polycythemia found in men living at high altitudes results from the anoxia brought about by the low oxygen tension in inspired air, acting on the erythropoietic organs which become hyperactive under such stimulation. Most investigators have observed in this polycythemia an increased percentage of reticulated red blood cells in the peripheral circulation, a finding which has been interpreted as evidence of hyperactivity in the formation of the erythrocytes. However, so far as can be determined no direct examination of the bone marrow tissues has been made in these permanent residents at high altitudes who have a reticulocytosis.<sup>1, 2, 3</sup> Thus, there is no direct evidence that reticulocytosis is paralleled by a corresponding hyperactivity in the blood-forming organs. Such a study is presented in this paper. The results also contribute to an understanding of the etiologic mechanisms underlying polycythemias classified as secondary and as polycythemia vera. Bone marrow studies in the former group have been made occasionally<sup>4, 5</sup> but the findings are not definitive. The following two factors emphasize the importance of investigating this problem: (1) the increase in circulating total blood volume, an alteration which commonly has been considered as characteristic of polycythemia vera,<sup>6</sup> also may be present to a high degree in secondary polycythemias,<sup>3, 9, 10</sup> and (2) an increase in leucocytes and platelets in the peripheral blood, as well as splenomegaly, may be absent in some cases of polycythemia vera. These considerations indicate the desirability of investigating the possible value of the bone marrow examination as an additional diagnostic criterion in the study of the polycythemic conditions.

### MATERIAL AND METHODS

Sixteen men of Indian race, permanent residents in Cerro de Pasco, a mining center located in the Peruvian Andes region at an altitude of 4,390 meters (14,400 feet), were studied. According to previous investigations carried out in Peru,<sup>1, 11</sup> the arterial oxygen saturation of men living at this altitude averages about 83.0 per cent. All the men had been born in this area or in nearby localities also situated at high altitudes. Their age varied between 15 and 60 years. Six of the men had worked in the mines for some time. All of them were apparently in good health but no radiologic examination was carried out to eliminate the possibility of an existing pneumoconiosis in the group of miners. One native within this group presented an abnormal degree of cyanosis.

In each case a sample of venous blood was taken and bone marrow tissue was aspirated after a sternal puncture. The venous blood sample was used in the following determinations: the number of red blood cells, white blood cells, and platelets per cubic millimeter, the hemoglobin in grams per 100 cc., and finally, the differential count of leucocytes. For this last

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analysis films, Wright stained, were used. For the hemoglobin determination a Sahli hemoglobinometer, previously calibrated by means of comparative determinations made with this instrument and the Van Slyke manometric apparatus, was employed. Platelets were counted following the technique described by Dameshek.<sup>12</sup>

The sternal puncture was performed at the level of the third intercostal space using a simple 16 gauge needle. About 0.5 cc of marrow material was aspirated,\* and fixed cover slip preparations were made promptly. The differential count of the bone marrow cells was made on the supravital preparations following the criteria established by Sabin and Miller<sup>13</sup> for cell identification. A total of 400 to 500 cellular elements was counted in each case.

### RESULTS OBTAINED

*A. Peripheral Blood*—Data obtained from the peripheral blood are summarized in Table I. The number of red blood cells varied between 5.41 and 7.67 millions per cubic millimeter, with a mean value of  $6.45 \pm 0.29$  millions, the hemoglobin had a mean value of  $19.4 \pm 0.69$  Gm per 100 cc, with extreme variations of 16.5 to 25.6 grams. These findings are in agreement with those obtained by Hurtado, Merino, and Delgado<sup>3</sup> in the study of native residents at Morococha, at an altitude slightly more elevated than Cerro.

TABLE I. HEMATOLOGIC STUDIES ON THE PERIPHERAL BLOOD OF 16 NATIVE RESIDENTS AT AN ALTITUDE OF 4,390 M (14,400 FT)

CASE	RED BLOOD CELLS (MILLIONS PER CMM)	HEMOGLOBIN (GM PER 100 CC OF BLOOD)	WHITE BLOOD CELLS (THOUSANDS PER CMM)	PLATELETS (THOUSANDS PER CMM)	PERCENTIC DIFFERENTIAL COUNT				
					NEUTROPHILES (%)	BASOPHILES (%)	EOSINOPHILES (%)	MONOCYTES (%)	LYMPHOCYTES (%)
1	6.48	18.2	6.60	259.00	45	1	5	6	43
2	5.89	19.0	6.85	176.55	55	1	2	9	34
3	7.67	25.6	6.45	210.10	55	0	4	10	31
4	7.22	24.5	6.80	100.00	57	0	12	2	29
5	6.64	19.2	4.50	477.08	40	1	2	2	55
6	6.88	20.2	6.75	172.00	64	0	1	8	27
7	5.41	16.5	10.65	481.05	47	1	3	5	44
8	7.04	19.2	6.55	527.63	54	0	5	6	35
9	6.81	20.7	5.30	190.54	37	0	1	3	59
10	6.58	20.4	5.85	315.60	50	0	3	6	41
11	7.37	21.0	7.00	397.71	60	0	7	9	24
12	6.25	17.6	6.85	293.75	61	0	0	4	35
13	5.43	16.5	4.50	314.94	63	0	6	4	27
14	6.27	17.3	8.20	386.63	46	0	2	2	50
15	6.48	17.7	11.00	621.60	38	0	2	3	57
16	5.87	16.8	6.50	440.25	40	0	11	7	42
Mean	6.45	19.4	6.86	335.22	50.7	0.3	4.1	5.3	39.6
± P.E.	±0.29	±0.69	±0.47	±36.85	±2.35	±0.10	±0.87	±0.67	±2.76
S.D.	1.13	2.68	1.83	143.90	9.1	0.4	3.4	2.6	10.7
± P.E.	±0.20	±0.48	±0.33	±26.26	±1.66	±0.07	±0.62	±0.47	±1.95
Coef. of var (%)	17.5	13.8	26.6	42.97	17.9	133.3	82.9	49.0	27.0
Extreme variations	5.41 7.67	16.5 25.6	4.5 11.0	100.8 621.1	37 64	0 1	0 12	2 10	24 59

\*Neutral red and Janus green stains were used in the preparation of the supravital films following the techniques in use at Dr. Charles A. Doan's laboratory (Ohio State University, Columbus, Ohio). We feel that the supravital preparations afford a greater advantage than the fixed Wright stained films in the differential cellular study because the characteristics of the cells are better preserved. For a critical study of this problem see Epstein, R. D. and Tompkins, E. H. Comparison of Techniques for Differential Counting of Bone Marrow Cells (Guinea Pig) Am. J. M. Sc. 206: 249, 1943.

The number of leucocytes and platelets were within the normal limits of variation. Mean values observed were  $686 \pm 0.47$  and  $335.2 \pm 36.8$  thousands per cubic millimeter, respectively. The differential leucocytic count also was found to agree with normal established values.

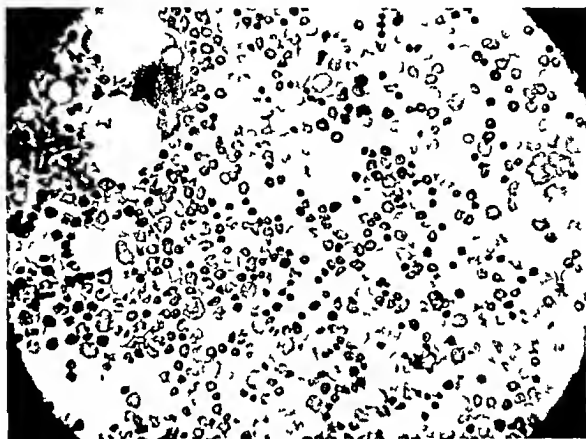


Fig 1—Photomicrograph of bone marrow tissue obtained by sternal puncture showing an increase in cellularity (Case )

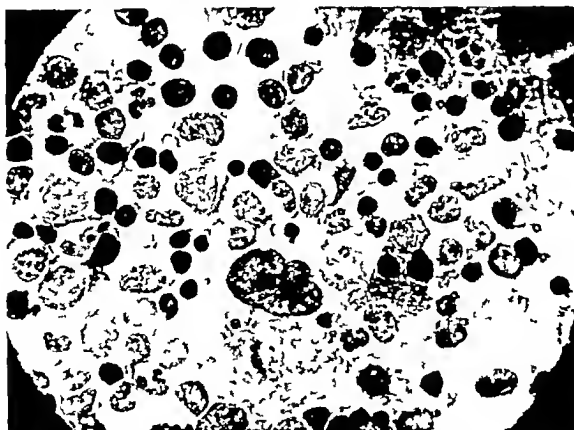


Fig —Photomicrograph of bone marrow (Case ) Note predominance of erythroid cells.

### *B Bone Marrow —*

*I Macroscopic Examination* A crude attempt to evaluate the degree of hyperplasia was made by examining macroscopically the number and size of marrow particles in the aspirated material. It is realized that such an estimation lacks precision, but a rough comparison could be made with marrow samples similarly collected from many normal persons and patients with various types of blood dyscrasias at sea level. The degree of estimated hyperplasia was graded from 1 to 3 plus. From this estimation, it was felt that the marrow was hyperplastic in ten men (62.5 per cent) and normal in six (37.5 per cent).

*II Microscopic Examination* A similar attempt was made to estimate the relative cellularity of specimens by scanning the flecks of marrow on four different supravital preparations with magnifications of 100 and 450. The same observer examined all preparations. The basis for comparison was again the experience gained from similar examinations at sea level. Estimated hyperplasia was arbitrarily graded as from 1 to 3 plus. The marrow was thought to be hypercellular in thirteen of the subjects (81.2 per cent) and normally cellular in three. In all the supravital preparations, amorphous and fatty material could be identified within the nests of hematopoietic tissue, but in only one instance (Case 10) was the increase in these substances striking. The degrees of hyperplasia as estimated by these two methods were not always in agreement. Samples which were regarded as normal macroscopically were often considered hypercellular when observed microscopically. No exact correlation, furthermore, could be established between the degree of polycythemia in the peripheral blood and the estimated hyperplasia of the marrow. Figs 1 and 2 are photomicrographs which illustrate the increased cellularity of the marrow specimens.

A more detailed study of the cellular composition showed that there was regularly an erythroid hyperplasia of sufficient degree to make the nucleated red blood cells more numerous than granulocytes. This change coupled with the fact that the great majority of nucleated red cells were at the normoblastic maturation level\* caused the marrows to resemble those seen in the hemolytic anemias<sup>5, 14, 15</sup> (Fig 3). In seven subjects (43.7 per cent) there was a moderate shift to younger cells of the erythroid series, early erythroblasts and megaloblasts were present in small numbers (Fig 4). Mitosis was frequently observed in the erythroid cells, but their number was not large.

The granulocytic elements were morphologically normal in all patients and showed no increase in younger forms of myelocytes. Polymorphonuclear leucocytes, metamyelocytes, and myelocytes "C" were the predominant elements in this series.

Megakaryocytes were seen in the thicker areas of the preparations. Most were mature in appearance, without qualitative changes and with multilobulated nuclei and a granular cytoplasm (Figs 1 and 2). None of the subjects showed the increase in megakaryocytes which so often is observed in polycythemia vera<sup>4, 5, 15-18</sup>.

\*The terminology of Sabin and Doan has been used throughout this paper for classifying the cells of both the erythroid and myeloid series.

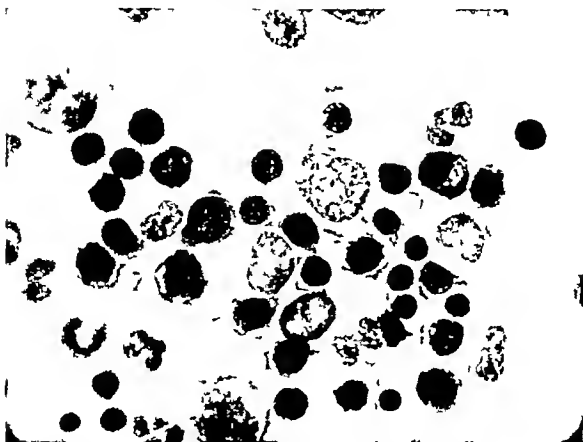


Fig 3—Bone marrow tissue (Case 13) Shows numerous nucleated red blood cells in greater proportion to other cellular elements.

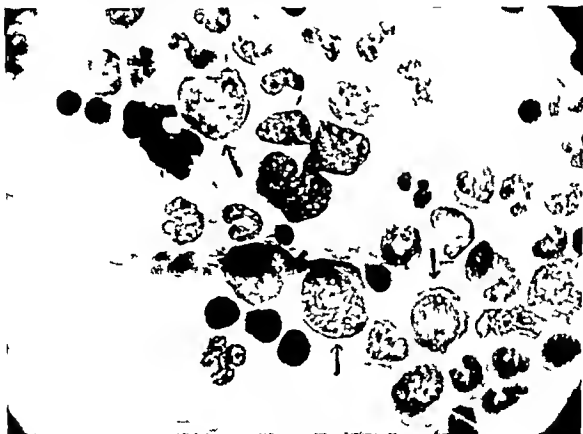


Fig 4—Bone marrow tissue (Case 1) Illustrates relative increase of young erythroblasts (marked with arrows)

Small lymphocytes were found in small numbers ( $0.6 \pm 0.14$  per cent) in nearly all of the samples examined but were less frequent than is usually accepted as normal<sup>6 15 27</sup> Clasmatoocytes with or without signs of phagocytosis, were observed in all cases but in only two instances (Cases 8 and 10),

TABLE II DIFFERENTIAL CELL COUNT IN BONE MARROW SAMPLES OBTAINED

CASE	MACROSCOPIC HYPERPLASIA	MICROSCOPIC HYPERPLASIA	NEUTROPHILES						BASOPHILES	EOSINOPHILES	
			SEGMENTED	BAND FORM	METAMYELOCYTES	MYELOCYTES "C"	MYELOCYTES "B"	MYELOCYTES "A"		SEGMENTED	MYELOCYTES
1	+++	N	10.5	4.1	9.2	11.9	1.3	0.2	0.9	1.0	1.3
2	N	+++	6.9	2.3	6.4	13.3	2.0	0.0	1.4	1.5	3.5
3	N	+++	4.8	2.5	10.6	15.8	1.2	0.2	0.4	1.0	1.5
4	N	+++	5.7	1.7	10.3	15.9	0.6	0.0	0.0	4.0	2.8
5	+++	+++	10.2	3.3	10.3	18.6	1.0	0.8	1.5	1.0	0.8
6	+++	+++	7.1	2.1	9.8	14.6	2.1	0.7	0.4	1.6	1.6
7	+++	+++	11.0	1.5	9.3	14.0	2.0	0.2	1.2	1.1	0.9
8	N	N	6.0	2.8	7.9	14.4	0.9	0.5	0.5	2.3	3.2
9	++	+++	13.2	4.0	12.7	16.2	1.9	1.4	0.3	0.0	1.2
10	++	N	5.3	1.5	6.0	11.5	1.1	1.1	0.0	3.8	1.1
11	++	+++	9.3	3.2	10.6	16.8	0.9	0.2	0.2	1.4	1.4
12	+++	+++	10.7	4.0	9.4	11.6	1.0	0.0	0.0	1.9	0.8
13	+++	+++	9.0	3.9	7.4	12.1	1.0	0.2	0.4	1.6	2.0
14	N	+++	14.3	2.5	11.9	12.2	0.8	0.0	0.5	3.1	1.0
15	++	++	11.2	4.2	8.0	10.9	1.3	0.7	0.0	1.5	0.4
16	++	+++	10.0	4.1	9.1	10.8	0.9	0.7	0.0	2.0	1.7
Mean			9.1	3.0	9.3	13.8	1.3	0.4	0.5	1.8	1.6
± P.E.			±0.94	±0.23	±0.46	±0.56	±0.06	±0.11	±0.12	±0.26	±0.18
S.D.			3.64	0.90	1.81	2.2	0.22	0.46	0.47	1.02	0.69
± P.E.			±0.66	±0.16	±0.33	±0.40	±0.04	±0.08	±0.08	±0.18	±0.12
Coef of var (%)			40	30	19	16	17	115	94	56	43
Extreme variations			5.3	1.5	6.0	10.8	0.6	0.0	0.0	0.0	0.4
			14.3	4.2	12.7	16.8	2.1	1.4	1.5	4.0	3.5

N Normal (as it is found in healthy people at sea level)

in which these cells averaged 5.1 and 4.2 per cent, was then number sufficiently large to be significant. Mature plasma cells, monocytes, and reticular cells were seen occasionally.

*III Differential Count of the Bone Marrow Cells* In all instances, the percentage of each type of cell (myelogram) was determined in the supravital preparations. From 400 to 500 cells were identified in each count. Table II gives the results obtained in this study. It is realized that the significance of a quantitative study of bone marrow cells in samples obtained from a single sternal aspiration is limited.<sup>15-20, 21</sup> However, if the interpretation is related to the general impression derived from a careful study of several preparations, such quantitative evaluation acquires a greater value. The predominant cells in the myeloid series were found to be myelocytes "C" ( $13.8 \pm 0.56$  per cent), metamyelocytes ( $9.3 \pm 0.46$  per cent), and segmented neutrophils ( $9.1 \pm 0.94$  per cent) (Table II). Comparison of these figures with those given for healthy men living at sea level indicates that the mean percentage of myelocytes found in our subjects agrees very closely with those given by Scott<sup>5</sup> and by Wintrobe.<sup>15</sup> 13.1 and 12.0 per cent, respectively. On the other hand, our mean percentage values for metamyelocytes and segmented neutrophils, 9.3 and 9.1 per cent, respectively, were lower than those found

IN 16 NATIVE RESIDENTS AT AN ALTITUDE OF  $\pm 390$  M (14,400 FT)

MYELOBLASTS	MONOCYTES	LYMPHOCYTES	CLASMATO CYTES		MEGAKARYO CYTES		PLASMA CELLS	NUCLEATED RED BLOOD CELLS			
			NO. MAL	PLASMOCYTIC	NO. NG	MATURE		NORMOBLASTS	LATE ERYTHROBLASTS	EARLY ERYTHROBLASTS	MEGAKYOBlasts
00	00	10	09	00	00	00	04	440	88	45	00
03	04	00	08	00	00	00	04	497	76	35	00
04	00	00	27	02	00	00	02	458	73	34	00
02	00	00	08	00	00	00	00	394	103	83	00
03	00	00	08	00	00	00	08	360	09	74	03
00	00	04	12	04	02	00	17	383	101	73	03
00	00	14	23	00	00	00	02	442	56	51	00
00	00	05	51	00	00	00	14	444	46	46	00
00	00	08	10	00	00	00	05	354	05	41	00
00	00	11	42	00	00	00	11	456	100	60	00
00	00	02	05	00	00	00	07	383	98	01	04
02	00	02	25	00	00	00	02	449	66	58	04
00	04	00	08	00	00	04	02	449	96	47	00
00	00	10	25	08	00	00	16	378	63	35	08
03	00	13	28	00	03	00	09	406	56	87	13
00	00	11	11	00	00	00	20	413	83	67	02
01 $\pm 0.01$	005 $\pm 0.03$	06 $\pm 0.14$	19 $\pm 0.34$	01 $\pm 0.05$	003 $\pm 0.002$	008 $\pm 0.03$	08 $\pm 0.13$	419 $\pm 1.05$	78 $\pm 0.45$	57 $\pm 0.43$	02 $\pm 0.00$
000 $\pm 0.01$	0.133 $\pm 0.02$	0.58 $\pm 0.11$	0.131 $\pm 0.23$	0.2 $\pm 0.04$	0.02 $\pm 0.01$	0.3 $\pm 0.04$	0.54 $\pm 0.11$	41 $\pm 0.74$	1.76 $\pm 0.32$	16 $\pm 0.30$	0.37 $\pm 0.08$
56	260	97	69	200	960	287	07	10	22	20	185
00 40	00 04	00 14	08 51	00 08	00 03	00 00	00 20	354 497	46 106	35 87	00 13

by the same investigators at sea level 15.7 and 14.8 per cent (Scott) and 20.0 and 22.0 per cent (Wintrobe). This relative decrease in the percentage of mature cells of the myeloid series cannot be interpreted as an indication of a shift to younger forms because the younger elements of this series myelocytes "A" and "B" and myeloblasts did not show a corresponding increase.

The most common cell in the erythrocytic series was the normoblast 41.9  $\pm 1.05$  per cent with variations between 35.4 and 49.7 per cent. This means percentage value found in the high altitude residents is definitely higher than that obtained by Scott and by Wintrobe<sup>13</sup> in the bone marrow of healthy adult subjects living at sea level 17.8 and 18.0 per cent respectively.

The level of polycythemia observed in the peripheral blood did not show a constant relationship to the degree of erythropoietic hyperplasia in the bone marrow or to the changes found in the proportion of the different erythrocytic cells in this organ. Calculation of the quantitative relationship between the erythroid and myeloid cellular elements (M/E ratio) gave the following mean percentage values:

Graanuloeytic series (myeloid)	40.9 per cent
Erythroeytic series	55.6 per cent
Other cellular elements	3.5 per cent
M/E ratio	1 to 1.4

	SEA LEVEL	HIGH ALTITUDE
MYELOID CELLS	75 0 %	40 9 %
ERYTHROID CELLS	20 0 "	55 6 "
OTHER CELLS	5 0 "	3 5 "

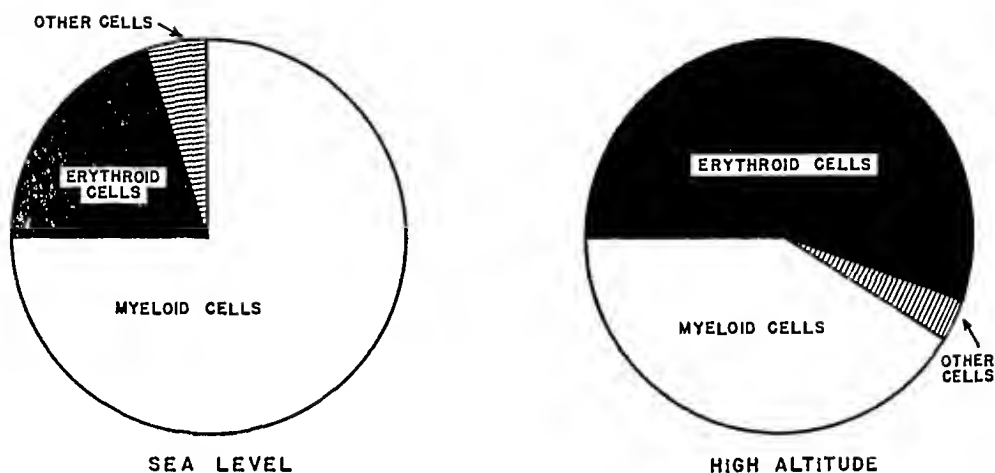


Fig 5—Graphic representation of the M/E ratio in bone marrow differential count made in residents at sea level and at high altitudes

These figures demonstrate that there is a very definite relative increase at high altitudes in the erythrocytic cells in the bone marrow, as compared with sea level (Figs 2 and 3). Our M/E ratio of 1 to 1.4 differs considerably from those of 3 to 1 or 4 to 1 considered as normal at sea level. A graphic representation of this observed ratio is given in Fig 5.

#### DISCUSSION

The observations just described indicate that there is an erythroid hyperplasia of the bone marrow in healthy people living permanently at high altitudes. The consistency of the results makes them seem valid even though the hazards of estimating hyperplasia from a single aspirated sample of marrow are recognized and admitted. The mean percentage value of 55.6 obtained for the erythrocytic elements, as compared with 40.9 per cent for the myeloid cells, in our subjects, is definitely higher than the corresponding values found at sea level by a number of different investigators. Doan,<sup>22</sup> 4 to 13 per cent, Manning,<sup>23</sup> 16.7 per cent, Mendell and others,<sup>24</sup> 15.4 per cent, Osgood and Seaman,<sup>25</sup> 17.2 per cent, Wintrobe,<sup>15</sup> 22.0 per cent, Piney and Hamilton,<sup>26</sup> 18 to 25 per cent, Young and Osgood,<sup>27</sup> 14.1 per cent, and Scott,<sup>5</sup> 17.8 per cent. Furthermore, as already has been indicated, our mean value of 1.0 to 1.4 for the M/E ratio in the high altitude subjects differs greatly from the ratios of 3 to 1 and 4 to 1 accepted as normal in healthy adults living at sea level.<sup>15, 25, 27</sup>



Quantitative study of the erythrocytic cells in the bone marrow showed a normal maturation process, with the normoblast being the predominant cell, while the more primitive ones, the erythroblasts and the megaloblasts, were found to be normal or only slightly increased. In consequence, the hyperplasia of the bone marrow in the high altitude subjects can be classified as normoblastic in nature or occasionally as normo erythroblastic.

It is interesting to compare our findings with those described in the studies made of the bone marrow in other cases of polycythemia, especially polycythemia vera. The bone marrow in this latter disease has been described as hyperplastic by some investigators<sup>4, 15, 17, 28</sup> while others such as Markoff, Noidenson, Robin Mallarme and Klum (quoted by Scott<sup>5</sup>) and Manning<sup>23</sup> concluded that increased cellularity is not a characteristic finding. In some of the cases of polycythemia vera which showed hyperplasia the process affected all cell elements of the bone marrow (polycythemia)<sup>4, 15, 28</sup>, the M/E ratio, therefore, was found to be normal or very little changed. In these instances the generalized hyperplasia appears different from the partial hyperplasia only erythrocytic in nature found in our cases of the polycythemia of high altitudes.

Other patients with polycythemia vera have been described as showing a definite hyperplasia of the erythrocytic cells of the bone marrow<sup>5, 8, 4</sup>, in these instances the proportion of nucleated red cells varied between 30 and 45 per cent of the number of cells counted and there was no evidence of a maturation arrest. These changes although similar in some respects to those observed by us in the polycythemia of high altitudes differ in other fundamental aspects. Most investigators agree that the bone marrow myeloid cells in cases of polycythemia vera show not only an increase in number but also an increase in younger forms such as young myelocytes and myeloblasts<sup>5, 15, 17</sup>. Furthermore, a remarkable increase in the number of megakaryocytes in the bone marrow of patients with polycythemia vera has been described as a frequent finding<sup>4, 6, 1, 18, 26</sup>. Those changes were absent in the subjects studied by us at high altitudes.

The results obtained in the present investigation agree closely with the observations made by Hurtado Meimo and Delgado<sup>3</sup> on the peripheral blood of a large number of permanent residents at high altitudes. These workers found no evidence of hyperactivity in the production of leucocytes. They indicated that anoxia appeared to be a specific stimulant for erythropoietic activity, leaving unaffected the formation of white blood cells. Our studies of the bone marrow cytology at high altitudes confirm that opinion and in addition emphasize the difficulties in accepting that concept that anoxia plays a fundamental etiologic role in the development of polycythemia vera.<sup>29</sup>

Schleicher<sup>30</sup> and Sundberg<sup>17</sup> have described volumetric variations in the different layers of the bone marrow and the latter investigator is of the opinion that the fat layer is nearly always absent in cases of polycythemia vera.<sup>31</sup> The supravital preparations in our cases always showed fat globules, but this observation cannot be used for comparison with the results in polycythemia vera.

Unfortunately, very few studies have been made on the cellular characteristics of the bone marrow in cases in which polycythemia has developed as a consequence of an anoxic condition brought about by respiratory or circulatory alterations. Zuntz, Loewy, Muller, and Caspary<sup>7</sup> and Dameshek<sup>4</sup> have reported cases of secondary polycythemia in which the bone marrow changes have been similar to those observed in polycythemia vera. On the other hand, Kienle<sup>6</sup> obtained results comparable to ours. It is desirable to have a greater number of observations in these cases in order to reach a better understanding of the value of sternal puncture in the differential diagnosis of the various types of polycythemia. Our study seems to indicate that at least the polycythemia of high altitudes, which is of secondary type, developing as a result of a known anoxic stimulus, differs in the cytologic characteristics of the bone marrow from most cases of polycythemia vera, a disease in which the etiologic mechanism is unknown.

#### SUMMARY AND CONCLUSIONS

The bone marrow of sixteen, healthy, Indian natives of adult age, permanent residents in Cerro de Pasco at an altitude of 4,390 meters (14,400 feet), was studied. Samples were obtained by means of sternal aspiration, and supravital and Wright stained preparations were used for the qualitative as well as quantitative microscopic examinations. All men showed a definite polycythemia in the peripheral blood.

The results obtained were compared with previous observations made in healthy people living at sea level and in patients with polycythemia vera.

1 The bone marrow of healthy, adult natives, living permanently at an altitude of 4,390 meters (14,400 feet), was interpreted as showing hyperplasia (hypercellularity) in 81.2 per cent of the subjects examined.

2 This hyperplasia was characterized by a marked increase of the cells of the erythroid series over the myeloid cells. Normoblasts were the most predominant cellular elements (normoblastic hyperplasia), and a reversed M/E ratio was a constant finding. The degree of hyperplasia was not always proportional to the level of polycythemia found in the peripheral blood.

3 Myeloid cells and megakaryocytes were not increased and the maturation process was normal in both the erythroid and myeloid series.

4 The foregoing findings seem to confirm further the opinion, previously expressed and derived from studies on peripheral blood, that anoxia acts as a specific stimulus for an increased activity in the formation of the erythroid cells leaving the myeloid cells unaffected.

5 A comparative study of our findings and those obtained in similar investigations made by several observers in cases of polycythemia vera seems to indicate that in general, the bone marrow cytology and the degree and kind of hemopoietic hyperactivity are not similar in the polycythemia of high altitudes and in polycythemia vera. This fact does not conform with the theory that anoxia is the underlying etiologic factor in the latter disease.

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## PLASMA TOCOPHEROL LEVELS IN VARIOUS PATHOLOGIC CONDITIONS

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THE blood concentration of vitamins depends not only on exogenous factors such as nutrition but also to a great degree upon endogenous processes such as diseases. Some evidence has been presented that the plasma level of the tocopherols reflects the nutritional status in that it is reduced in malnutrition<sup>1</sup> and sprue<sup>2</sup> but not in fibrositis<sup>3</sup>. The effect of disease upon the plasma tocopherol is not established. The influence of various diseases and especially those of the liver upon the metabolism and plasma level of vitamin A has been the subject of intensive investigations<sup>4-5</sup>. It has been shown that in the acute stage of hepatic disease the plasma vitamin A is lowered, less as the result of faulty intestinal absorption than of deficient release of vitamin A from the liver. This theory is based upon fluorescence microscopic findings which in hepatic damage revealed displacement of vitamin A from its original sites within the liver cell to abnormal locations<sup>6</sup> and upon chemical analyses which suggested a defect in hepatic vitamin A esterase<sup>7</sup>. The plasma vitamin A level can thus, under certain circumstances, become an index of hepatic function. The question arose as to whether the metabolism of vitamin E as another fat soluble vitamin A is subject to similar influences by the liver. This question is of significance since vitamin E has a protecting influence upon the liver in carbon tetrachloride intoxication<sup>8</sup> and in experimental nutritional injury<sup>9-10</sup>. Moreover, the recently claimed beneficial effect of vitamin E in vascular diseases<sup>11</sup> makes such a study desirable.

The plasma tocopherol level in various normal and abnormal conditions, both before and after the intake of a test dose of tocopherol, was determined. It was compared with the plasma vitamin A alcohol and ester. In addition, the concentration of tocopherol in human liver and bile under normal and pathologic conditions was determined. This study was facilitated by the recent development of methods for the chemical determination of tocopherols in organs and plasma<sup>12-14</sup>.

### MATERIAL AND METHODS

Tocopherol in serum and bile was determined by the method of Quayle<sup>12,13</sup> and in the liver by the methods of Himes<sup>14</sup> with Quayle's modifications for colorimetric assay. The plasma vitamin A was determined by the method of Kimble<sup>15</sup>, the partition, according to the recently used modification of Gillam<sup>16</sup>. Plasma tocopherol was determined before breakfast on nine normal subjects (technicians, interns, nurses) and seventy one patients suffering from various diseases. In ten instances, the determination was repeated three,

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six (or seven), and twenty four hours after the intake of 1,500 mg of mixed tocopherol.<sup>17</sup> The time interval was chosen from experiences with the response of the plasma vitamin A level to the intake of large doses of the vitamin.<sup>17</sup> The bile was obtained from patients in whom, during operation, biliary drainage was established. It was collected after biliary secretion appeared normal as to amount and appearance. The liver specimens were obtained at autopsy.

## RESULTS

As Table I shows, the results in normal subjects agreed well with the reports in the literature. In miscellaneous diseases the range was significantly widened. In nephritis the level as a rule was much higher than in normals, in nephrosis, less so. In heart diseases the level was consistently higher than in normals and the highest level found occurred in this group. In liver diseases without biliary obstruction as in infectious hepatitis or cirrhosis without jaundice, the level was slightly but not significantly lower than in the normals. In obstructive jaundice, it was though not regularly higher than in normals. In one case of xanthomatous biliary cirrhosis it was only slightly elevated. In patients with toxic hepatitis and cirrhosis with jaundice it was slightly higher than in normals but in some it was low. In carcinoma other than that of the biliary tract there was a tendency for the level to be slightly higher than normal. One case of malnutrition showed normal values.

TABLE I. TOCOPHEROL CONCENTRATION IN THE PLASMA OF NORMAL SUBJECTS AND OF PATIENTS SUFFERING FROM VARIOUS DISEASES

DIAGNOSIS	NUMBER OF CASES	TOCOPHEROL CONCENTRATION IN MG/100 CC PLASMA		
		AVERAGE	MAXIMUM	MINIMUM
Normals	9	0.94	1.38	0.51
Miscellaneous diseases	7	1.13	2.40	0.15
Nephritis	5	1.55	1.92	1.02
Nephrosis	4	1.16	1.68	0.81
Heart diseases	9	1.78	2.52	1.20
Infectious hepatitis	9	0.87	1.86	0.45
Toxic hepatitis	5	1.36	1.83	1.17
Cirrhosis without jaundice	6	0.92	1.94	0.39
Cirrhosis with jaundice	14	1.26	2.28	0.51
Obstructive jaundice	7	1.61	2.51	0.36
Carcinoma other than in biliary tract	5	1.43	1.62	0.78

A plot of the tocopherol level with the plasma vitamin A level failed to reveal a significant correlation (Fig 1). There was also no correlation between plasma level of tocopherol and that of vitamin A alcohol or ester.

TABLE II. TOCOPHEROL CONCENTRATION IN PLASMA AND BILE

DIAGNOSIS	TOCOPHEROL IN MG/100 CC	
	BILE	PLASMA
Stricture of hepatic duct	0.65	
Choledocholithiasis	2.80	
Choledocholithiasis	0.76	0.90
Choledocholithiasis	1.40	1.17
Choledocholithiasis	0.74	1.29

Kindly supplied by Hoffmann-La Roche Inc. Nutley N. J. as Ephynal Acetate (d 1 alpha tocopherol acetate) tablets of 50 milligrams.

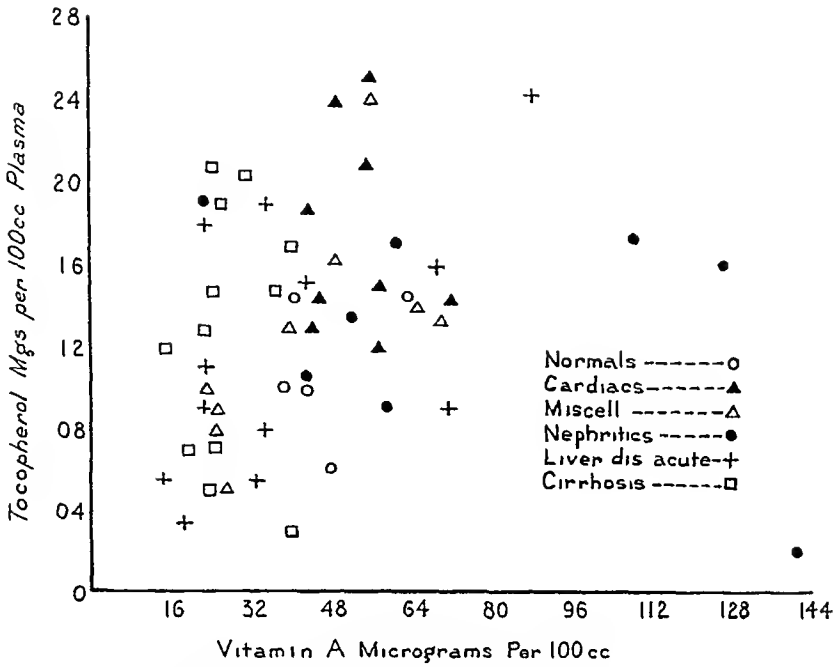


Fig 1—Plot of the plasma tocopherol level against the plasma level of total vitamin A

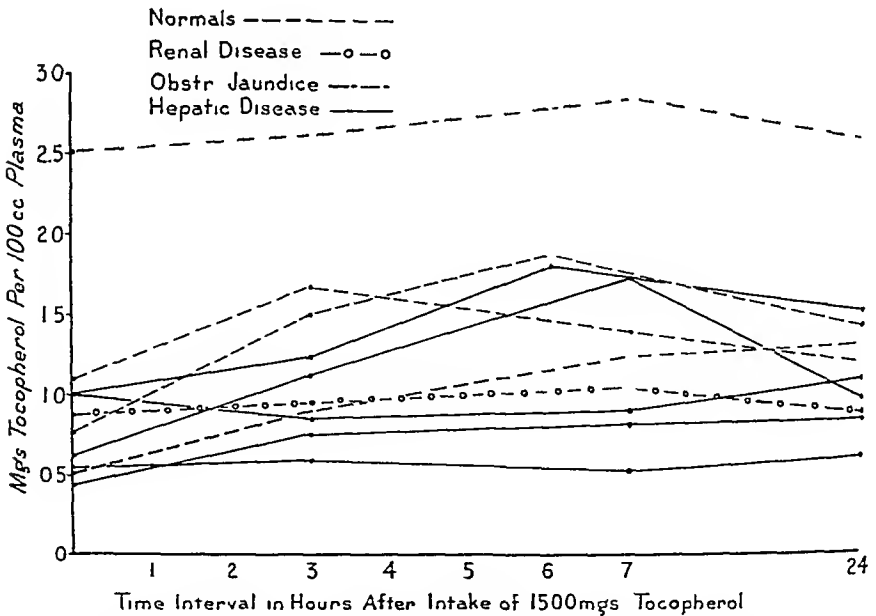


Fig 2—Response of the plasma tocopherol level to the intake of 1500 mg mixed tocopherol in oil in normals and in patients suffering from various diseases

The bile obtained from patients with operative bile fistulas revealed tocopherol concentrations of about the same range as the plasma (Table II). In the three instances in which both were determined at the same time on the same patient, there was a moderate parallelism.

After intake of a large test dose the plasma tocopherol level rose significantly and regularly in normals, whereas in patients with acute hepatitis or cirrhosis the rise was, though not regularly, depressed or absent. In one case of obstructive jaundice the level rose significantly from a high fasting level. In one patient with nephrosis, the rise was insignificant (Fig. 2).

In two patients with liver diseases the tocopherol concentration of the liver was significantly lower (Laennec's cirrhosis 0.441 mg per 100 Gm, obstructive jaundice, 0.944 mg per 100 Gm) than in two others dying from diseases not related to the liver (carcinoma of colon 2.639 mg per 100 Gm, cor pulmonale 3.374 mg per 100 Gm).

#### DISCUSSION

The variations of the plasma tocopherol level in pathologic conditions reveal no correlation with those of the plasma vitamin A level. The significant and characteristic decrease of the plasma vitamin A level in liver diseases and other severe conditions does not hold true for the tocopherol level. The latter reveals a slight tendency to be lower in pure hepatocellular disease but a definite tendency to be higher in obstruction of the biliary tract. This is also reflected in the elevated levels in cirrhosis with jaundice. This behavior parallels that of cholesterol which is also elevated in some hepatobiliary diseases, supposedly because of impaired biliary excretion. However, the concentration of tocopherol in the bile does not exceed its plasma concentration in contrast to cholesterol. Other factors therefore, have to be considered for the elevation of the plasma tocopherol level in obstructive jaundice. Its slight reduction in hepatocellular damage could be in keeping with the significantly lowered tocopherol concentration in the liver, in nonhepatic diseases the range of the hepatic tocopherol concentration agrees with that reported in animals.<sup>1</sup> Whether poor intestinal absorption of tocopherol in oil—already previously demonstrated in cirrhosis<sup>1</sup>—is the only factor explaining the low hepatic stores in liver diseases remains to be established.

The plasma tocopherol level is elevated in a variety of diseases e.g. carcinoma, even if not involving the biliary tract. The elevation in nephritis in contrast to nephrosis parallels the behavior of vitamin A but not of cholesterol.<sup>1</sup> The rather constant elevation in cardiac diseases does not speak for a vitamin E deficiency in such conditions. The latter has been assumed as rationale for the treatment of heart diseases with high doses of vitamin E. The elevation of the plasma tocopherol in pathologic conditions requires further study which should be associated with the clarification of the biochemical function of vitamin E as an antioxidant.

#### SUMMARY

1. The variations of the plasma level of tocopherol in various diseases do not parallel those of vitamin A and therefore the metabolism of vitamin E is influenced by endogenous factors in a different fashion from that of vitamin A.

2 Plasma tocopherol is only slightly and not consistently reduced in hepatocellular damage. It is increased in obstructive jaundice, carcinoma, nephritis (but not nephrosis), and most consistently in cardiac diseases.

3 The tocopherol concentration of the bile shows a moderate parallelism to that of the plasma.

4 The normal use of the plasma tocopherol following the intake of large doses of tocopherol in oil is often absent in liver diseases.

5 In the few instances studied, the hepatic tocopherol concentration was lower in patients with liver diseases than in those dying from other diseases.

6 The reduction of the plasma tocopherol in liver diseases may be related to the factors mentioned. Its elevation in various other diseases is thus far not explained.

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## EVALUATION OF THE FLOCCULATION TEST WITH HAYEM'S SOLUTION

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**D**IAGNOSTIC problems as they arise in the care of jaundiced patients often call for more or less elaborate examinations which are not readily available to the average physician.<sup>1,3</sup> Hence, a simple serum reaction with Hayem's solution, reported to be a useful aid in the management of hepatic disorders<sup>2,4,10</sup> appeared to be worthy of study. With an appraisal of its practical value as the chief aim, the test was applied to a general hospital population and its results correlated with pertinent clinical and laboratory findings.

### MATERIAL AND METHODS

In initial experiments titration of fresh serum with Hayem's solution by daylight as prescribed in Gros' method<sup>1,7</sup> was found to yield inconsistent results. The two end points "reversible" and "irreversible" flocculation, differed in the same specimen with different observers, with varying intensities of illumination, and with changes in the rate of titration. Because of these and other shortcomings, the method was modified and standardized, as follows. Equal volumes of serum and the reagent (usually 1 ml. of each) are mixed, a procedure first employed by Jacobson<sup>17</sup>, development of a precipitate ("irreversible flocculation") within one to twenty-four hours is considered a positive reaction which may be graded as one, two, and three plus according to the amount of precipitate. An immediate prediction of the eventual outcome of the test can usually be made by first overlaying the serum with 1 drop of Hayem's solution while a faint transient clouding is seen with proper artificial lighting in any (normal) serum a well discernible "reversible flocculation" is an abnormal finding ("Gros reaction"). The results of this combined Gros-Jacobson or Hayem's (H) test, as designated hereafter are consistent and unaltered by accidental hemolysis or prolonged refrigeration of the serum but may be interfered with by heavy lipemia. The Hayem's solution used in this study contains 1.0 per cent sodium chloride according to the Swiss Pharmacopoeia and as described by Gros.<sup>6</sup> The solution generally employed in this country containing 0.5 per cent sodium chloride was found by us to produce precipitates in the sera of obviously healthy persons and consequently to be of no diagnostic value. There is no difference however in the value of either reagent in the performance of red blood cell counts.

The H test was performed on sera from 550 clinic and hospital patients. As a rule, either serum left over after the routine performance of the Kahn test or part of a fasting blood sample drawn for other diagnostic procedures was used. These included in 300 patients, one or more of the tests listed in Table I. The modification of the Takata-Ara procedure employed<sup>20</sup> was chosen because it allows grading of the reaction more readily than the original technique.<sup>21</sup> Weak reactions (flocculation beyond the second test tube) were included among the positive results. "Gamma globulin" as referred to in the tables and text, signifies the results of the precipitation method using ammonium sulfate.<sup>9</sup> This method

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TABLE I PATHOLOGIC LEVELS CHOSEN IN BLOOD STUDIES PERFORMED

TEST	PATHOLOGIC VALUES
Erythrocyte sedimentation rate <sup>18</sup>	Above 20 mm per hr in men Above 25 mm per hr in women
Cephalin cholesterol flocculation <sup>19 20*</sup>	Above 2 plus (after 48 hr)
Thymol turbidity <sup>21 23†</sup>	Above 49 units
Zinc sulfate turbidity <sup>24†</sup>	Above 12 units
Formol gel <sup>25</sup>	Gelation
Takata Arr <sup>26, 27</sup>	Precipitate in first 3 or more tubes
Total serum globulin <sup>28†</sup>	Above 3.0%
Albumin globulin ratio <sup>28†</sup>	Below 1.3
"Gamma globulin" <sup>29†</sup>	Above 1.4%

\*Difco Laboratories Detroit Mich

†Photometer No 606 Cenco Central Scientific Co Chicago Ill

‡Photocolorimeter No 313 D Lutz Inc New York N Y

yields an average of 85 per cent of the true gamma fraction. The borderline between abnormal and normal results of all the comparative tests was drawn so as to include only definitely pathologic reactions in the first group, mostly in accordance with recommendations by other authors (Table I).

#### RESULTS AND COMMENT

The significant clinical and laboratory findings in 300 patients are listed in Table II. Two hundred fifty volunteers, employment examinees, and patients admitted for orthopedic conditions or for elective surgery served as a control group in which the H reaction was consistently found to be negative.

Hyperglobulinemia and high gamma globulin fractions were prevalent in the group of H-positive sera (Table II, A). This fact and a similar parallelism in results with the other studies listed seem to illustrate the mechanism of the H test. The chemically related Takata procedure showed the best agreement though lower sensitivity—in accordance with observations made by other authors comparing the Takata with the Glos test.<sup>5, 6, 10, 11, 14, 15</sup> Formation of a precipitate in a mixture of serum with a solution of mercury bichloride evidently depends on the presence of high-molecular protein fractions.<sup>5, 6, 10, 21, 26, 30, 32</sup>

Gamma and total globulin levels, known to be major factors in all the reactions studied and especially in the zinc sulfate test,<sup>15, 19, 21, 26, 30, 43</sup> were found to be moderately raised in some H-negative sera (Table II, B). Apparently, the increase must be considerable in order to produce an H flocculation, in contrast to the highly sensitive zinc sulfate turbidity. Hence, a mild hepatitis or an early stage of the disease may not be expected to influence the H test.<sup>24, 31, 32, 39, 44, 48</sup> A return to normal of a formerly positive reaction may herald recovery. This, indeed, was our experience in many instances of primary hepatic and other disorders, such as thyrotoxicosis and acute and chronic infectious processes.

There are many disorders "not primarily hepatic"<sup>49</sup> in which the plasma gamma globulin is commonly found to be augmented.<sup>32, 33, 37, 46, 47, 50, 51</sup> They include all those in which a positive H test was encountered, save for thyrotoxicosis.<sup>32, 46, 47, 55</sup> In all of them the occurrence of positive sedimentation and flocculation tests has been reported.<sup>3, 27, 30, 32, 36, 38, 39, 42, 43, 47, 49, 56, 60</sup> and is again demonstrated in this study. Used singly as a screening method, any one of them,

including the H test, would essentially be a potential indicator of one thing only—organic disease, one of the many causing physicochemical plasma alterations. The lines drawn by the various procedures, and the size and character of the diagnostic groups encircled by them, differ according to the finer mechanism of each test in which qualitative changes of the gamma fraction, other globulin alterations in the albumin and lipid contents may play a role.<sup>4 25 30 43 46 47</sup>

In our material, the Takata and Hanger tests would appear to be the least sensitive ones, separating only 12.1 and 13.5 per cent respectively, of 300 cases as "disease suspects." Next in relative sensitivity was the formal gel test picking up 16.5 per cent, then the thymol turbidity and Hayem tests (both about 26 per cent), the sedimentation rate (51.5 per cent), and, finally, the zinc sulfate turbidity test (62.6 per cent). There was variation not only in number but also in type of cases among those "groups of suspects," as exemplified by the incidence of primary hepatic disease in each. Of the group defined by a positive Takata reaction, about 30 per cent had either hepatitis or cirrhosis; of the group with a positive cephalin cholesterol flocculation, 48 per cent, of the positive formal gel group, 21 per cent, of the positive Hayem's, 22 per cent, of the positive thymol turbidity 49 per cent, of the elevated sedimentation rate, 10 per cent, and of the group with a positive zinc sulfate turbidity 18 per cent. Taking all the cases of primary hepatic disease encountered, the percentage of positive reactions was 20 with the use of the formal gel test, 35 with the Takata-Ara method, 58 with the cephalin cholesterol flocculation, 63 with the H test, 75 with the sedimentation rate, 78 with the thymol, and 89 with the zinc sulfate turbidity test. Hence, in grading these procedures as to their relative value in detecting hepatic disorders, the formal gel and Takata tests would be on the bottom and the two turbidity reactions on top of the scale. Since all these tests except the cephalin cholesterol flocculation, yielded positive results in the later stages of obstructive jaundice (Table II, A) they cannot be relied upon, singly for differentiation between "medical" and "surgical" jaundice.<sup>1 3 27 31 38 57 68</sup> While, in this regard, the cephalin cholesterol flocculation exhibited the greatest diagnostic selectivity, the H test appeared to possess prognostic value since it pointed out the most severe forms of hepatitis and all cases of cirrhosis, as well as the more prolonged and complicated instances of biliary obstruction.

A positive H flocculation occurred in all cases of active rheumatoid arthritis, in 10 per cent of the cardiac group, in 51 per cent of acute infectious diseases, in 47 per cent of chronic lung disorders, and in 59 per cent of malignant neoplasms. It implied systemic illness usually denoting the more serious and/or advanced case and was more ominous than the abnormal outcome of the routine sedimentation test at times even coinciding with a normal rate. There were no "false positive" reactions. In some instances a positive H test was the first abnormal sign demonstrable followed sooner or later by the detection of a major illness such as cirrhosis, syphilis, pyonephrosis, pleuritis,

TABLE II CORRELATION OF HAYEM'S TEST WITH OTHER BLOOD STUDIES AND CLINICAL FINDINGS

NUMBER OF PATIENTS	DIAGNOSIS	NUMBER OF ABNORMAL RESULTS										CLINICAL FINDINGS
		SEDIMENTATION RATE	OLIPHAN CHOLESTEROL FLOCC	TUBERCULIN BIDITY	ZINC SULFATE TUR BIDITY	FOR MOUL GEL	TAKATA AREA	TOTAL GLOBULIN	A/G RATIO	"GAMMA GLOBULIN"		
<i>A Patients With Positive Hayem's Test</i>												
6	Hepatitis	3 <sub>4</sub>	4	4 <sub>6</sub> (3)	4 <sub>4</sub> (2)	1 <sub>3</sub>	0 <sub>4</sub>	3 <sub>5</sub>	3 <sub>5</sub> (2)	3 <sub>5</sub> (3)	4 cases of infectious, 2 of homologous serum jaundice. Severe illness and protracted course	
11	Cirrhosis	8 <sub>9</sub>	7 <sub>10</sub>	6 <sub>8</sub> (5)	7 <sub>7</sub> (4)	1 <sub>3</sub>	7 <sub>9</sub>	8	11 (10)	6 <sub>6</sub> (2)	1 case of postinfectious type, 1 Laennec's Jaundice in 8, as cited in 5	
3	Obstructive jaundice	3	0	1	3 (1)	3	2	3	3 (2)	2 (1)	1 case of stenosis of common duct, 2 of carcinoma of pancreas. Duration 2 to 6 months	
8	Rheumatoid arthritis and scleroderma	8	3	4 <sub>6</sub> (3)	6 (2)	3 <sub>4</sub>	4 <sub>7</sub>	6 <sub>7</sub>	6 <sub>7</sub> (6)	5 <sub>5</sub> (3)	2 cases of active infectious, 5 of rheumatoid arthritis, 1 of advanced scleroderma	
5	Heart disease and thyrotoxicosis	4	2	0 <sub>1</sub>	2 <sub>2</sub>	0 <sub>1</sub>	0 <sub>4</sub>	1 <sub>4</sub>	1 <sub>4</sub>	1 <sub>4</sub>	2 cases of severe hyperthyroidism, 1 of cardiovascular and neurosyphilis, 2 of severe passive congestion due to arteriosclerotic (pulm edema) and rheumatic heart disease (cardiac cirrhosis?), respectively	
20	Acute infectious disease	15	3	3 <sub>1</sub> (1)	11 <sub>11</sub> (3)	2 <sub>5</sub>	3 <sub>16</sub>	6 <sub>16</sub>	11 <sub>10</sub> (9)	8 <sub>8</sub> (3)	7 cases of pneumonia, 1 pleuritis, 2 of lung abscess, 1 rheumatic fever, 1 brucellosis, 3 primary syphilis, 1 pyonephrosis, 1 malaria, 1 pancreatitis, 2 cellulitis of legs. Severe illness in all but syphilis patients	
15	Chronic lung disease	14	2	2 <sub>6</sub>	6 <sub>6</sub> (4)	0 <sub>2</sub>	5 <sub>13</sub>	8 <sub>14</sub>	7 <sub>11</sub> (6)	3 <sub>2</sub> (1)	12 cases of active pulm tuberculosis, moderately to far advanced, 2 of tuberculous pleuritis, 1 bronchiectasis	
10	Neoplastic disease	8	1	1 <sub>6</sub>	5 <sub>6</sub>	1 <sub>2</sub>	2 <sub>1</sub>	2 <sub>8</sub>	4 <sub>5</sub> (3)	2	3 cases of carcinoma of bronchus, 1 of breast, 1 testis, 1 thyroid, 1 pancreas, 1 colon, 1 lymphoblastoma, 1 leucemia. Widespread metastases in 8 cases, downhill course in all	
78	Abnormal results	85%	30%	44 6% (25 5%)	93 5% (32 6%)	42 3%	37 1%	54 5%	67 6% (55 0%)	100% (43 4%)		

B. Patients With Negative Hayem's Test

	4 <sub>1</sub>	4	8 (1)	5 <sub>1</sub>	1 <sub>1</sub>	0 <sub>1</sub>	2	4 <sub>1</sub>	
10 Hepatitis									5 cases of homologous serum, 5 of infectious jaundice. Early stage in 4, later stage but benign course in 6
8 Gall bladder disease and obstructive jaundice	5	0 <sub>1</sub>	1	1 <sub>1</sub> *	1 <sub>1</sub> *	0	1	0	3 cases of jaundice of less than 1 mo duration due to carcinoma in 1, calculus in 2. Abnormal results indicated by refer to case of repeated past attacks of jaundice, due to stone
45 Heart disease	13	2 <sub>1</sub>	2 <sub>1</sub>	12 <sub>1</sub> (1)	1 <sub>1</sub>	0 <sub>1</sub>	2 <sub>1</sub> (2)	2 <sub>1</sub>	19 cases of hypertension, 19 of arteriosclerotic, 5 of rheumatic, 2 of syphilitic heart dis, with congestive failure in 17, myocard infarction in 5 pulmonary infarct in 3, prostatic disease in 5, etc
19 Acute infectious disease	14	3 <sub>1</sub>	2 <sub>1</sub>	6	0 <sub>1</sub>	0 <sub>1</sub>	3	1	10 cases of pneumonia, 1 of rheumatic fever, 2 primary syphilis, 3 tonsillitis, 1 treated malaria, 1 mumps, 1 cellulitis of hand. Mild illness or convalescing stage
17 Chronic lung disease	8	0	0	2	0 <sub>1</sub>	0 <sub>1</sub>	1 <sub>1</sub> (1)	0	11 cases of pulm. tuberculosis with 4 active far advanced, 7 quiescent or arrested, 6 of chronic bronchitis and/or asthma
7 Neoplastic disease	6	0	0	2 <sub>1</sub> (1)	0 <sub>1</sub>	0	1	1	1 case of carcinoma of bronchus, 1 of breast, 3 stomach, 2 prostate. All ran stationary course though mostly incurable by surgery
116 Miscellaneous	30 <sub>1</sub>	0 <sub>1</sub>	3 <sub>1</sub>	17 <sub>1</sub> (1)	0 <sub>1</sub>	0 <sub>1</sub>	5 <sub>1</sub>	2 <sub>1</sub>	27 cases of peptic ulcer, with secondary anemia in 10, 6 gastritis or enteritis, 14 diabetes, 16 alcoholism, 13 or ganic renal insufficiency (NPN 0.000 238 Gm per cent), osteoarthritis, prostatic, common cold dermatoses etc
222 Abnormal results	40%	0.6%	17.4% (11.1%)	47.7% (3.0%)	5.1%	0%	2.2%	10.5% (1.2%)	
300 Total abnormal results in A and B	51.6%	13.5%	26.4%	62.6%	16.5%	12.1%	19.9%	29.8%	

Inferior numbers indicate that the test was not performed in all patients in that group they represent the number of patients in whom it was performed

Numbers in parentheses refer to thymol turbidity reactions above 10 units zinc sulfate turbidity reactions above 0 units A/G ratios below 0

active tuberculosis, metastatic neoplasm. The test was helpful in such differential diagnostic questions as early obstructive or hepatic jaundice, tumor of the right kidney or hepatomegaly, epistaxis due to nasal lesion or to chronic hepatic disease, cholecystopathy or neoplastic liver involvement, "rheumatism" or rheumatoid arthritis, congestive or infectious pleural effusion, nutritional or leucemic anemia. A positive reaction favored, a negative one was against, the hepatic, infectious, or neoplastic disorder under consideration. While in such problems the quantitative analysis of the blood proteins would represent a more accurate index,<sup>2, 3, 23, 29, 32, 37, 46, 47, 50, 55</sup> the quick orientation afforded by the test proved to be valuable and seldom misleading.

A correlation of all positive H reactions with a significant increase in gamma globulin could either be demonstrated or, clinically, postulated, except for the two cases of hyperthyroidism. The results found in this condition point to a rise in serum alpha globulin and/or a fall in albumin<sup>32, 46, 47, 55</sup> as possible additional factors in the mechanism of the H test. A negative H reaction does not seem to rule out any of the ailments summarized, with the exception, perhaps, of cirrhosis of the liver and active rheumatoid arthritis—if such conclusions are permitted on the basis of the small number of such cases studied.

It appears reasonable to include the H test in the category of serologic "liver function" tests. While all these reactions reflect serum protein changes which are common diagnostic features of liver disease, they are to be attributed largely to the general phenomenon of reticuloendothelial irritation quite independent of liver function.<sup>1, 3, 23, 31, 32, 36, 39, 57</sup> Since the morbid processes in this group, including those enumerated, constitute a major part of everyday practice, the chief value of the H test seems to lie in its nonspecific screening capacity, calling attention to the presence of organic disease, much like the sedimentation rate but quite different in scope. When used as a quick office procedure, it is apt to point out the more severe forms of those conditions, it may further provide ready supplementary evidence and serve as a prognostic guide. While in the diagnosis of liver disease it appears qualitatively inferior to some of the standard tests in use, the clear-cut objective results of the H reaction and the simplicity in technique, requiring no instrument, constitute practical advantages. These facts, and the general availability of the reagent should favor the adoption of the test as a routine clinical procedure.

#### SUMMARY

Results of a serum flocculation test with Hayem's solution (H test) were compared with those of standard flocculation reactions, erythrocyte sedimentation rate, and serum protein fractionation.

Of 550 persons screened with the test, all of the 78 (14 per cent) who showed a positive flocculation had a systemic illness of either metabolic, infectious, or neoplastic nature. Similar diseases occurring in some of the H-negative cases were usually milder or less advanced. Serum globulin alterations,

as reflected by the comparative procedures, appeared to be the major factors producing a positive reaction.

In the diagnosis of liver disease, the II test was found to be less specific than some of the other flocculation tests but was superior to most of them by consistently yielding a positive result in all instances of hepatic emulosis. A similar observation was made in active rheumatoid and infectious arthritis.

The test is recommended as a simple office procedure which can provide diagnostic information comparable to that obtained with more elaborate flocculation reactions.

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## THE COLLOIDAL RED TEST AS AN INDEX OF LIVER DYSFUNCTION

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THE colloidal gold test as modified by MacLagan<sup>1</sup> is recognized as a valuable test in the study of liver dysfunction. However, the preparation of the gold sol is time consuming and tedious, these facts have restricted the wider clinical application of the test. In 1947, Ducci<sup>2</sup> recommended a procedure in which a modified scarlet red solution is used and which is carried out following the general principles of MacLagan's colloidal gold reaction. The preparation of this solution is simple and the suspension is more stable than the gold sol. In the present clinical study, the colloidal red test was compared with two other recognized methods of a similar category, namely, cephalin-cholesterol flocculation and thymol turbidity, in 122 subjects. These included twenty normal individuals, eleven patients with infectious hepatitis, seven with portal cirrhosis, eleven with chronic cholangitis, twenty-two with chronic cholecystitis and cholelithiasis, seven with malignancy of the gastrointestinal tract and liver metastases, and forty-four patients with miscellaneous disorders.

### METHODS

The cephalin-cholesterol flocculation test was carried out as introduced by Hanger<sup>3</sup> and modified by Neefe and Rheinhold.<sup>4</sup> Values exceeding 2+ flocculation were interpreted as abnormal. The thymol turbidity test was performed by the method of MacLagan.<sup>1</sup> The procedure was modified by adding 0.1 cc of serum to 6 cc thymol buffer solution. Values exceeding 4 units were considered abnormal. The colloidal red test was carried out according to the procedure outlined by Ducci.<sup>2</sup> Values exceeding 2+ flocculation were considered abnormal.

### RESULTS

Table I shows the results obtained in twenty normal individuals free of liver or biliary tract disease. It will be noted that in no instance was the colloidal red flocculation greater than 2 and this occurred in only two of the twenty subjects.

Table II details the results in eleven cases of infectious hepatitis (epidemic and homologous serum varieties). It will be noted that the colloidal red agreed with the cephalin-cholesterol flocculation in nine instances. In one of the remaining two cases (Patient 1), the cephalin-cholesterol and thymol turbidity tests were negative while the colloidal red was 3+. In the other (Patient 5), cephalin-cholesterol flocculation was 3+ while the other tests were normal. Thymol turbidity compared favorably with the colloidal red test in eight of the eleven patients. In the other three, the thymol turbidity reaction was within normal limits while the colloidal red precipitation was definitely abnormal.

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TABLE I NORMAL SUBJECTS

SUBJECT	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOIDAL RED FLOCCULATION
		24 HR	48 HR		
1	7.0	±	±	2.0	0
2	8.8	±	±	1.5	0
3	10.0	0	0	1.0	0
4	10.0	0	0	0.5	0
5	7.5	1+		1.0	2
6	9.3	±	±	1.0	1
7	8.3	±	±	2.0	0
8	6.8	1+		1.0	1
9	5.7	0	0	2.0	0
10	6.1	0	0	1.5	0
11		0	±	1.0	0
12	8.8	0	0	0.5	0
13		±	±	1.0	0
14		±	±	1.5	0
15	8.8	0	0	1.5	0
16	5.6	1+	1+	1.5	1
17	7.5	1+	1+	1.0	0
18	6.0	±	±	1.0	0
19	5.7	0	0	1.0	0
20		1+	0	3.5	0

TABLE II PATIENTS WITH INFECTIOUS HEPATITIS

PATIENT	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOIDAL RED FLOCCULATION
		24 HR	48 HR		
1	12.5	2+	2+	2.0	3
2	17.0	4+	4+	12.0	5
3	11.5	3+		2.0	3
4	8.3	3+	3+	2.0	3
5	45.0	3+	3+	4.0	2
6	12.5	0	±	1.0	1
7	8.3	±		2.5	2
8	39.5	3+	3+	10.0	5
9	37.5	3+	3+	5.0	4
10	57.5	3+	3+	8.0	5
11	18.6	4+	4+	5.0	5

Table III shows the results in seven cases of portal cirrhosis. The cephalin cholesterol flocculation and colloidal red tests agreed in five instances. In the remaining two cephalin cholesterol flocculation was normal while colloidal red precipitation was abnormal. Thymol turbidity and colloidal red precipitation compared favorably in five of six cases. In the remaining instance thymol turbidity was normal whereas the colloidal red precipitation was abnormal.

TABLE III PATIENTS WITH PORTAL CIRRHOSIS

PATIENT	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOIDAL RED FLOCCULATION
		24 HR	48 HR		
1	15.0	±	±	3.0	3
2	15.0	4+	4+	8.0	5
3	24.0	2+		5.0	5
4	6.8	3 4+			4
5	1.0	3 4+		7.5	3
6	8.3	1+	1+	1.0	0
7	37.5	3+	3+	5.0	5

Table IV details the results in twenty-two cases of chronic cholecystitis with stones. Two subjects (Patients 14 and 17) had associated common duct stones verified at operation. In none of these was a positive colloidal red test obtained. In one instance (Patient 17), the cephalin-cholesterol test was 3+, thymol turbidity 5 units, and the colloidal red flocculation at the upper limit of normal.

TABLE IV PATIENTS WITH CHOLECYSTITIS AND CHOLELITHIASIS

PATIENT	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOIDAL RED FLOCCU- LATION
		24 HR	48 HR		
1	4.0	±		2.0	±
2	5.4	0		1.0	0
3	6.7	0		2.0	0
4	5.5	0		2.0	0
5	5.0	0		1.0	0
6	3.0	0		1.0	0
7	3.9	0		1.0	0
8	5.0	±		3.0	1
9	4.0	0		1.0	0
10	5.0	1+		0	0
11	5.0	0		2.3	2
12	5.3	2+	2+	2.0	1
13		1+	1+	0.5	0
14	250.0	0	0	0.5	0
15	6.2	2+	2+	2.0	0
16	8.8	0	±	2.0	2
17	20.6	3+		5.0	2
18	5.9	0		4.0	0
19	39.5	±	±	2.0	1
20		0		1.2	2
21		0		2.0	0
22	12.5	±		1.0	1

Table V indicates the results in eleven cases of chronic cholangitis. In six patients the cholangitis was due to bacterial infection (*Escherichia coli*). In eight, there was good agreement between the cephalin-cholesterol flocculation and the colloidal red tests. In the other three, cephalin-cholesterol flocculation was normal while the thymol turbidity and colloidal red tests were abnormal.

TABLE V PATIENTS WITH CHRONIC CHOLANGITIS

PATIENT	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOIDAL RED FLOCCU- LATION	ORGANISM	ASSOCIATED CLINICAL CONDITION
		24 HR	48 HR				
1		0		2.0	0	<i>Esch. coli</i>	
2	11.2	3+		5.0	3		
3	4.1	3+		5.6	1	<i>Esch. coli</i>	
4	5.8	2+		10.0	3	<i>Esch. coli</i>	Biliary cirrhosis
5	3.0	3+		20.0	4	<i>Esch. coli</i>	Subclinical hepatitis
6		0		9.0	4	<i>Esch. coli</i>	Stenosis, common duct
7	125.0	0	0	2.0	0	<i>Esch. coli</i>	Biliary cirrhosis
8		2+		15.0	4		
9		0		4.0	0		
10	93.8	1+	1+	1.0	0		
11		1+		4.0	1.2		Intrahepatic duct ob- struction

The thymol turbidity and colloidal red reactions agreed in ten of the eleven cases. In the remaining one both the cephalin cholesterol and thymol turbidity tests were abnormal whereas the colloidal red precipitation remained normal.

Table VI shows the results in seven cases of malignancy of the gastrointestinal tract with secondary metastases to the liver. In only one patient was an abnormal flocculation test observed. A 3+ colloidal red reaction. Apparently the colloidal red test is of no greater value in detecting carcinomatosis of the liver than the other flocculation tests.

TABLE VI PATIENTS WITH SECONDARY CARCINOMA OF THE LIVER

PATIENT	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOIDAL RED FLOCCULATION	LOCATION OF PRIMARY LESION
		-48 HR	+48 HR			
1		2+	-+	4.0	0	Colon
2	83.0	1+	1+	4.0	1	Gall bladder and duodenum
3	121.0	0	±	0.5	0	Pancreas
4	3.6	0		2.0	2	Common duct
5		0		1.0	0	Stomach
6	25.0	1+	1+	3.0	3	Ascending colon
7	60.0	0	0	2.0	2	Head of pancreas

Table VII details the findings obtained in forty-four patients with various diseases. It will be noted that in ten instances an abnormal colloidal red test was obtained. These included two patients with scleroderma, two with diabetes mellitus, one with chronic rheumatoid arthritis, one with chronic arthritis due to gout, one with thyrotoxic heart disease, one with schistosomiasis, one with chronic alcoholism, and one with duodenal ulcer. In patients with scleroderma, schistosomiasis, and rheumatoid arthritis, an augmented precipitation of colloidal red might be expected because of the associated hyperglobulinemia. Again, patients with diabetes mellitus, thyrotoxicosis, and chronic alcoholism might have associated parenchymal cell damage. The abnormal colloidal red test in one patient with duodenal ulcer remains unexplained.

#### DISCUSSION

The biochemical mechanism responsible for the flocculation of the cephalin cholesterol, thymol, and colloidal red suspensions remains unexplained. In the absence of serum albumin, electrophoretically pure gamma globulin flocculates with the standard cephalin cholesterol reagent. The minimum quantity of gamma globulin necessary to produce precipitation is said to be between 0.22 and 0.11 milligram<sup>6</sup>. Kabat and co-workers<sup>7</sup> as well as Moore and associates<sup>8</sup> detected no difference between gamma globulin from normal or hepatitis sera. On the other hand, MacLagan and Bunn<sup>9</sup> found that hepatitis gamma globulin was more effective in flocculating cephalin cholesterol emulsions at a pH of 7.8 than normal gamma globulin. The sensitivities were 0.05 mg and 0.5 mg, respectively. They also found that a mixture of alpha and beta globulins from patients with hepatitis flocculated the cephalin cholesterol reagent while these

TABLE VII PATIENTS WITH MISCELLANEOUS DISORDERS

PATIENT	DIAGNOSIS	ICTERUS INDEX (UNITS)	CEPHALIN CHOLESTERYL FLOCCULATION		THYMOL TURBIDITY (UNITS)	COLLOID AL RED FLOCCU LATION
			24 HR	48 HR		
1	Subacute pancreatitis		0		4.0	0
2	Subacute pancreatitis		0		2.0	0
3	Chronic pancreatitis	4.0	0		2.0	0
4	Acute pancreatitis	14.6	2.3+		5.0	4
5	Common duct sphincter spasm	4.4	0		2.0	0
6	Common duct sphincter spasm	4.8	0		2.0	0
7	Common duct sphincter spasm	4.0	0		2.0	0
8	Common duct sphincter spasm	5.2	0		2.0	±
9	Scleroderma		4+		10.0	5
10	Scleroderma	5.0	2+	2+	3.0	3
11	Scleroderma	10.0	1+	1+	1.0	0
12	Scleroderma	6.2	±	±	0.5	0
13	Hypertensive cardiovascular disease	7.5	1+	2+	2.0	0
14	Arteriosclerotic heart disease, cerebro spinal syphilis	8.3	1+	1+	2.0	0
15	Arteriosclerotic heart disease	12.5	1+	1+	0.5	0
16	Arteriosclerotic heart disease		0		1.0	0
17	Syphilitic heart disease	7.5	0	0	1.0	0
18	Rheumatic heart disease	12.5	±	±	2.0	1
19	Myocardial infarction	6.0	±	±	1.0	0
20	Congestive heart failure	30.0	1+	1+	1.0	0
21	Hypertensive cardiovascular disease, post-sympathectomy	15.0	0		1.0	1
22	Thyrotoxic heart disease	7.1	2+	2+	1.5	3
23	Recurrent thyrotoxicosis	9.3	0		1.5	0
24	Chronic rheumatoid arthritis	6.2	2+	2+	3.0	5
25	Gouty arthritis	7.1	±	±	3.5	4
26	Osteoarthritis, spine	5.0	0		2.0	0
27	Gastrointestinal bleeding	8.8	1+	1+	1.0	1
28	Duodenal ulcer	5.0	0		2.0	1
29	Duodenal diverticulum	6.0	0		3.0	1
30	Chronic atrophic gastritis	5.3	±	±	1.5	1
31	Duodenal ulcer		1+	1+	1.0	0
32	Duodenal ulcer	17.4	2+		2.5	4
33	Diabetes mellitus	4.5	±		20.0	3
34	Diabetes mellitus, hepatomegaly	11.5	1+	1+	2.0	0
35	Stenosis of hepaticoduodenostomy open ing		1.2+		3.4	2
36	Addison's disease	7.3	1+		3.0	2
37	Pernicious anemia	5.5	3+		1.0	1
38	Chronic alcoholism	7.5	±	±	2.0	3
39	Migraine	5.2	0		4.0	2
40	Anatomic defect of gall bladder	3.8	0		1.0	0
41	Spastic colon	4.9	0		1.0	0
42	Seborrheic dermatitis	10.0	1+	1+	1.0	0
43	Hookworm infestation of the small bowel	5.3	±	±	2.5	1
44	Schistosomiasis	6.9	3+		5.0	5

fractions from normal sera did not. Moore and co-workers<sup>6</sup> reported that inhibition of flocculation occurred when 5.9 mg of normal albumin were added to the reagent containing 0.33 mg of gamma globulin. A similar quantity of hepatitis albumin only reduced the flocculation from 4+ to 3+. MacLagan and Bunn<sup>7</sup> found that normal albumin was inhibitory to both normal and

hepatitis gamma globulin fractions in the proportion of 100:1. Thus, it appears that the flocculation of the cephalin cholesterol suspension depends mainly on some relationship between the gamma globulin and albumin fractions of the serum.

The mechanism responsible for the thymol turbidity reaction appears to be different. According to Melagran and Bunn<sup>9</sup> and Recant, Chaigaff, and Hanger,<sup>10</sup> precipitation of the thymol reagent depends upon the presence of a lipid containing fraction of the serum. Cohen and Thompson<sup>11</sup> found by electrophoretic analysis that the thymol reagent precipitates beta globulin from both normal and hepatitis serum. Maclagan and Bunn<sup>8</sup> demonstrated that gamma globulin produced a faint turbidity when added to the thymol reagent, the turbidity increased markedly, however, when cephalin or lecithin was added. They found the turbidity abolished by normal but not by hepatitis albumin. Thus, although the thymol turbidity reaction involves primarily the beta globulin fraction of the serum, there is some evidence that gamma globulin may also be involved but to a lesser degree.

Electrophoretic analysis of the serum proteins responsible for the precipitation of the colloidal red reagent has as yet not been reported, although such studies have been made on the colloidal gold reaction upon which this procedure is based. The gold sol like the cephalin cholesterol reagent, is precipitated by gamma globulin and the precipitation is inhibited by serum albumin.<sup>9</sup> Alpha and beta globulin mixtures have been reported to be even more inhibitory than albumin.<sup>9</sup> The present clinical study does not permit the formulation of any definite statement as to the possible mechanism responsible for the precipitation of the colloidal red suspension. It is interesting to note that this report includes several instances wherein the colloidal red test was definitely abnormal while the cephalin cholesterol and thymol turbidity reactions were within the normal range.

It should be emphasized that the tests used in this study are indicators of changes in the blood protein fractions. They mirror primarily alterations in gamma globulin and to a variable degree albumin and beta globulin. Because changes in these protein fractions are an accompaniment of hepatic disease, these tests are of value as indices of hepatic damage. Positive flocculation tests are not always the result of hepatic disease as noted in Table VII.

The fact that positive flocculation tests occur in hepatic disease only when the parenchymal cell is damaged makes them useful as an aid in the differential diagnosis of jaundice (see Tables II, III and VI). The colloidal red test like the cephalin cholesterol flocculation and thymol turbidity reactions is normal in early extrahepatic (major bile duct) obstruction i.e. before secondary damage to the liver cells has occurred by interference with bile flow.

In summary it may be stated that the colloidal red reaction is a sensitive test for determining alterations in the serum protein fractions resulting from damage to the parenchymal cells of the liver. Its technical simplicity recommends it for routine use. This statement does not imply that the colloidal red test should replace the other flocculation procedures. In fact, these tests supplement rather than supplant each other. Obviously the simultaneous performance of the three flocculation procedures described in this paper offers a

better panorama of the underlying alterations in the serum protein fractions than can possibly be surmised by the use of any single procedure alone<sup>12, 13</sup>

#### SUMMARY

1 A clinical evaluation of the colloidal red flocculation test is presented. In 122 subjects the test was compared with two other widely used procedures of a similar type, namely, the cephalin-cholesterol flocculation and thymol turbidity tests.

2 This study reveals that the colloidal red test is a sensitive and technically simple procedure for detecting alterations in the serum protein fractions accompanying hepatic disease. Its use as an aid in the differential diagnosis of jaundice is recommended.

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# THE INFLUENCE OF DIBENAMINE UPON CIRCULATORY REACTIONS TO EPHEDRINE AND NEOSYNEPHRINE IN NORMAL MAN

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ONE of the drugs most recently employed to produce blockade of the sympathoadrenal system is Dibenamine\* (dibenzyl  $\beta$  chloroethylamine). Nickerson and Goodman<sup>1</sup> have reported on its efficacy in preventing the vasopressor response to epinephrine and the excitatory response of smooth muscle to sympathetic nerve stimulation as well as to epinephrine in several organs of the body. They indicate, in addition, that the inhibitory effects of epinephrine are not influenced by the drug.

Different experimental methods have been employed to demonstrate the important protection that Dibenamine exerts against the cardiac effects of epinephrine in animals anesthetized with cyclopropane<sup>1, 2</sup> as well as those not subjected to anesthesia during the course of study.<sup>3, 4</sup> Although the site of action of this drug is not definitely established, the available evidence suggests that Dibenamine acts directly upon effector cells to prevent the responses to sympathin E and the excitatory manifestations of epinephrine.<sup>1, 5</sup>

Because of these interesting properties of Dibenamine, this study was undertaken to determine its effects on the actions of two commonly employed vasopressor drugs: ephedrine and Neosynephrin.

## METHODS

The vasomotor responses to ephedrine sulfate or Neosynephrin hydrochloride as manifested by changes in blood pressure, pulse rate, digital pulsations, and finger volume were measured in normal human subjects before and after Dibenamine administration, permitting each to act as his own control. Ephedrine was studied in this manner in three subjects and Neosynephrin in three others.

All tests were carried out approximately two hours after meal time in a dimly illuminated room at a temperature of 26 to 29° C. The subjects were observed in the supine position on a comfortable table after a slow intravenous infusion of physiologic saline solution had been started. Measurements of blood pressure were made by auscultation using a mercury sphygmomanometer with a pneumatic cuff applied to the right upper arm. The digital pulsations, pulse rate, and changes in finger volume were recorded on a Turner plethysmograph<sup>6, 7</sup> with the cup covering the distal phalanx of the left index finger.

A period of psychic and physical rest was allowed until the functions under observation had reached a steady state. These values for blood pressure and pulse rate are given in Table I. The absolute values for finger volume and amplitude of digital pulsations are of less importance.<sup>7</sup> Normally there are gradual variations in both these values. Changes to be significant should be sudden, reproducible, and related to known causes.

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TABLE I. CHOLATORY RESPONSES TO EPHEDRINE AND NEOSYNERGIN BEFORE AND AFTER DIBENAMINE\*

TRIAL DRUG	SUBJECT*	DIBENAMINE (MG PER KG)	INITIAL† BLOOD PRESSURE	INITIAL† PULSE RATE	SYSTOLIC PRESSURE (MM HG)	DIASTOLIC PRESSURE (MM HG)	PULSE RATE (PER MIN)	FINGER VOLUME (CC)	DIGITAL PULSATIONS (CC PER 5 CC)
Ephedrine	1 A		130/80	78	Incr 53	Incr 22	Deer 10	Deer 0.07	Deer 0.005
	B	4	132/82	88	Incr 30	Incr 8	Incr 6	Deer 0.08	Deer 0.007
Ephedrine	A		110/64	76	Incr 42	Incr 24	Deer 40	Deer 0.04	Deer 0.011
	B	5	118/70	88	Incr 22	No change	Irreg Incr 16	Incr 0.20	No change
Ephedrine	A		120/76	60	Incr 36	Incr 15	Deer 4	Deer 0.03	Deer 0.006
	B	5	124/80	68	Incr 16	Incr 8	Incr 4	Deer 0.12	Deer 0.006
Neosynephrin	A		130/68	88	Incr 100	Incr 50	Deer 28	Deer 0.22	Deer 0.010
	B	4	128/62	84	Incr 20	Incr 10	Irreg Deer 2	Deer 0.04	Deer 0.004
Neosynephrin	A		150/90	80	Incr 76	Incr 40	Deer 12	Deer 0.06	Deer 0.032
	B	5	125/60	92	Incr 15	Deer 4	Incr 8	Incr 0.04	Incr 0.015
Neosynephrin	A		100/58	80	Incr 68	Incr 32	Deer 36 Irreg Deer	Not recorded	Deer 0.011
	B	5	102/62	80	Incr 10	Incr 8	Deer 18	Not recorded	Deer 0.004

\*A Before the administration of Dibenamine B After the administration of dibenamine

†After a period of psychic and physical rest

Without the subject's knowledge the test drug, ephedrine (37 mg) or Neosynephrin (15 to 20 mg), was then injected intravenously and the effects were recorded. A day or two later Dibenamine hydrochloride applied as a 5 per cent solution in 50 per cent alcohol, was administered to the subjects in doses of 4 to 5 mg per kilogram of body weight in an infusion of 300 to 500 cc of physiologic saline solution over a period of thirty to eighty minutes. With rapid administration nausea was seen in Subject 3. One person (Subject 6) suffered a burning pain and tenderness along the course of the vein receiving the infusion until the Dibenamine was diluted to 600 cc and local heat was applied. Within three hours of the completion of the administration of Dibenamine the observations described were repeated. In Subject 2, observations were recorded only ten minutes after the completion of the Dibenamine infusion and the control series was performed nine days later.

### RESULTS

When ephedrine was given intravenously in the control series the blood pressure increased within thirty to sixty seconds reaching a maximum in three to six minutes. The systolic pressure increased 36 to 53 mm and the diastolic pressure 15 to 24 mm of mercury. Both returned to original levels within twenty minutes. Coincident with the highest blood pressure there was a reflex slowing of the pulse of 8 to 40 beats per minute. Then as the blood pressure fell, the pulse rate increased 8 to 15 beats above the original level gradually returning to normal. Subject 2 exhibited an irregular pulse rhythm characterized by pulsus alternans during the hypotension. Finger volume was decreased by 0.03 to 0.07 cc within three minutes and gradually returned to the original size. The amplitude of digital pulsations was decreased by 0.005 to 0.011 cc per 5 cc of finger mass within three minutes after the ephedrine injection and returned to normal in ten minutes.

After Dibenamine had been administered the responses to intravenous ephedrine were reduced. The increase in systolic pressure was 10 to 30 mm and the diastolic pressure 0 to 16 mm mercury. No bradycardia was noted. There was an increase in pulse rate (4 to 18 beats per minute) within six minutes and it remained elevated for more than fifteen minutes. There were no episodes of pulse irregularity. In Subjects 1 and 3 the finger volume was decreased by 0.08 and 0.13 cubic centimeter. In Subject 2 there was a gradual increase in finger volume. The amplitude of digital pulsations decreased by 0.007 cc per 5 cc in Subjects 1 and 3 but remained unchanged in Subject 2. These results are epitomized in Table I.

In the control trials after Neosynephrin was given intravenously the systolic pressure was increased by 68 to 100 mm Hg within one to four minutes and the diastolic pressure increased 34 to 50 mm Hg in the same time. Both systolic and diastolic pressures returned to original levels in fifteen minutes. The pulse rate was decreased by 12 to 34 beats per minute coincidentally with the highest blood pressure. Only Subject 5 showed a secondary rise in pulse rate as the blood pressure fell. With the slowest bradycardia pulse irregularities consisting of extrasystoles were exhibited by Subjects 4 and 6. The finger volume was decreased by 0.22 cc and 0.07 cc in subjects 4 and 5 and returned to normal within five to eleven minutes. The amplitude of digital pulsations was decreased by 0.011 to 0.032 cc per 5 cc finger mass and returned to the original levels in eight to ten minutes.

After the administration of Dibenamine, Neosynephrin no longer produced such dramatic effects. The rise in systolic pressure was only 10 to 20 mm mercury. Diastolic pressure rose 10 and 8 mm Hg in Subjects 4 and 6 and fell 4 mm Hg in Subject 5. The pulse rate rose slightly in Subjects 4 and 5 but fell 18 beats per minute in Subject 6. There were no instances of irregular pulse rhythm. Finger volume did not change appreciably. The amplitude of digital pulsations decreased in Subjects 4 and 6 and increased in Subject 5. These results are summarized in Table I.

#### DISCUSSION

It will be seen that with the doses used, Dibenamine hydrochloride almost completely prevented the vasopressor effects of Neosynephrin. Peripheral vasoconstriction, as evidenced by a decrease in finger volume and decrease in the amplitude of digital pulsations, was minimized also.

The vasopressor effects of ephedrine were mitigated but not abolished by Dibenamine. Increases in blood pressure, while lower after Dibenamine, were still appreciable. Digital vasoconstriction in response to ephedrine was not prevented in two out of three trials (Subjects 1 and 3). In Subject 2, digital vasoconstriction was prevented by Dibenamine. An explanation may be found in the fact that the test was performed only ten minutes after the Dibenamine infusion had been completed.

These findings indicate the existence of different modes of action for ephedrine and Neosynephrin. Others<sup>1, 2, 3</sup> have demonstrated that Dibenamine completely abolished the pressor effects of epinephrine. Ephedrine, according to Gaddum,<sup>3</sup> acts by inhibiting amine oxidase, a ubiquitous enzyme, which destroys sympathin. The action of sympathin is thus potentiated. However, if the action of ephedrine was mediated through sympathin, Dibenamine should prevent its pressor actions. That it does not do so is evidence against this postulate.

Since the vasopressor and vasoconstrictor effects of ephedrine are not completely abolished by Dibenamine, ephedrine should be more effective than either epinephrine or Neosynephrin in producing these results in patients to whom Dibenamine has been administered.

#### SUMMARY

Circulatory responses to ephedrine and Neosynephrin were observed in man before and after the administration of Dibenamine hydrochloride.

The vasopressor and vasoconstrictor actions of Neosynephrin are almost completely prevented by Dibenamine. The vasopressor effects of ephedrine are decreased and the vasoconstrictor effects are not appreciably affected by Dibenamine.

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#### DISCUSSION

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#### SUMMARY

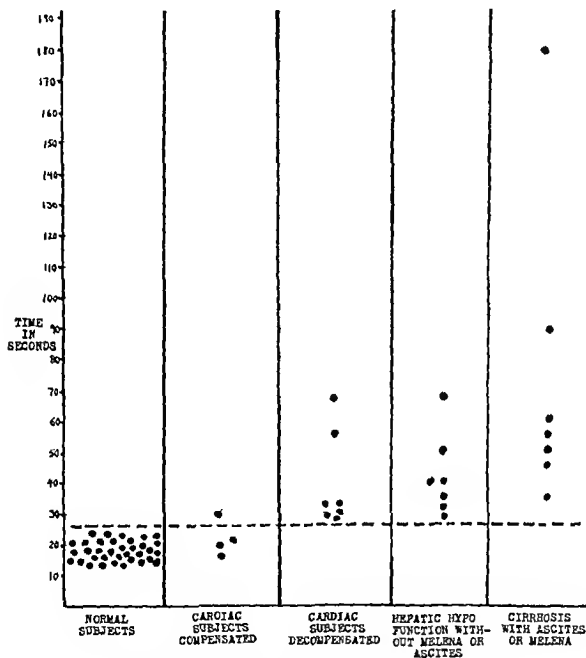
Circulatory responses to ephedrine and Neosynephrin were observed in man before and after the administration of Dibenamine hydrochloride.

The vasopressor and vasoconstrictor actions of Neosynephrin are almost completely prevented by Dibenamine. The vasopressor effects of ephedrine are decreased and the vasoconstrictor effects are not appreciably affected by Dibenamine.

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technique is that the end point is completely subjective and will vary with different observers, although for each observer a normal range can be secured. A more objective technique utilizing radioactive gas, is being investigated. However, the present method has the advantage of not requiring any special apparatus or training and can be used at the bedside by the general practitioner.



CROSS PORTAL CIRCULATION TIME IN FIVE GROUPS OF PATIENTS

Fig 1

#### RESULTS

In thirty four normal controls and three compensated cardiac subjects the cross portal circulation time ranged between 11 and 25 seconds. The span in seven decompensated cardiac subjects and in one clinically compensated cardiac patient was between 32 and 65 seconds. Seven patients with Laennec's cirrhosis with clinical evidence of portal hypertension evinced by either melena or ascites but without evidence of cardiac failure gave measured times between 34 and 180 seconds.

A group of fourteen cases of miscellaneous types of hepatic disease were studied. None of these subjects had melena or ascites or cardiac failure but all showed hepatic hypofunction by the usual liver function tests. Half of these subjects presented gross portal circulation times lower than 25 seconds, while the remaining seven had prolonged times between 27 and 65 seconds. This latter group may represent cases of portal hypertension without clinical manifestations. Prolonged gross portal circulation time due to heart failure can be readily differentiated from that of portal cirrhosis by the prolonged arm-to-tongue circulation time in the former.

#### SUMMARY

A simple method for the quantitative estimation of portal circulation time is described.



# ACTINOMYCES BOVIS IN TISSUES AND BODY FLUIDS

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## INTRODUCTION

THIS work is being presented to show the incidental finding of *Actinomyces bovis* in diseases other than actinomycosis. The study was prompted by the discovery of *A. bovis* in a bone marrow aspiration from a suspected case of brucellosis which was determined later to be Hodgkin's disease. Consequently, various materials such as blood and bone marrow, from all subsequent cases of Hodgkin's disease, brucellosis or related syndromes were cultured for this organism. Routine bone marrow aspirations from patients with various blood dyscrasias were cultured as controls. *A. bovis* was recovered from specimens of seven patients. The presence of this organism in the bone marrow or blood of individuals suffering from a disease other than actinomycosis has not previously been reported.

We are prompted to publish this preliminary report because of the scarcity of proper material for study at this institution. Further investigation at this and other laboratories will test the value of our findings.

## METHODS

The media used were products of Difco and consisted of brain heart infusion, Tryptose broth, Tryptose phosphate broth and thioglycollate broth. The latter was distributed in 20 cc quantities in tubes and the others in 60 cc quantities in flasks.

Inoculation of media with the body fluids and tissues was as follows. Bone marrow: Two milliliters of bone marrow were added to 3 ml of 2.5 per cent sodium citrate in distilled water. One milliliter of this mixture was added to each of the flasks of different media and 0.5 ml to each of two tubes containing thioglycollate broth. Blood: Fifteen milliliters of blood were added to 3 ml of 2.5 per cent sodium citrate solution and of the mixture 4 ml quantities were added to the flasks of different media and 1 ml to each of two tubes of thioglycollate broth. All other specimens were inoculated *ad libitum*.

Incubation was carried out at a temperature of 37° C. The brain heart infusion cultures were kept in the normal atmosphere and the Tryptose broth cultures (for cultivation of *Brucella abortus*) were maintained in an atmosphere of increased carbon dioxide. Specimens inoculated into the Tryptose phosphate broth were grown under anaerobic conditions. Of the two tubes of thioglycollate broth seeded with each specimen one was grown in an anaerobic atmosphere and the other in an atmosphere of increased carbon dioxide.

## CASE HISTORIES

CASE 1 (No. 4328) — A clinical problem diagnosed as brucellosis. The Brucella skin sensitivity reaction and the brucella agglutination tests were negative initially but the latter became positive (1 to 640) after the skin test and subsequent vaccination with brucella antigen. Eleven specimens of blood, one of bone marrow and one of spinal fluid were cultured for brucella and other organisms. Of these one blood culture, the bone marrow specimen, and the spinal fluid showed *A. bovis*. The diagnosis of Hodgkin's disease was made on necropsy findings.

From the Laboratory Service of the Veterans Administration Hospital.

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CASE 2 (No 8739) —Brucellosis and Hodgkin's disease were the diagnoses considered. Agglutination tests for brucella were negative. Twelve specimens of blood, two of bone marrow, one liver biopsy specimen, four sputa, and one sample of gum scrapings were cultured. Of these, *A. bovis* was recovered from one blood specimen, the two bone marrow samples, and the liver biopsy. The final clinical diagnosis of Hodgkin's disease was confirmed by the liver biopsy.

CASE 3 (No 8823) —Clinically diagnosed as Hodgkin's disease, confirmed histologically. One specimen of blood and one of bone marrow were cultured, the latter showing the presence of *A. bovis*.

CASE 4 (No 8635) —An ophthalmologic problem, diagnosed as nummular keratitis, most probably due to brucellosis. Brucella agglutination, Brucellergen skin test, and opsonic index failed to confirm Brucella as the etiologic factor. Two blood specimens, two bone marrow aspirations, and corneal scrapings were bacteriologically studied. *A. bovis* was recovered from the two bone marrow aspirations and the corneal scrapings.

CASE 5 (No 9340) —Clinical diagnosis of xanthoma tuberosum. In the routine study of bone marrow, a culture also was done and *A. bovis* was recovered.

CASE 6 (No 9936) —Clinically diagnosed as abdominal granuloma. Culture of a bone marrow aspiration revealed the presence of *A. bovis*.

CASE 7 (No 9912) —Clinically diagnosed as Hodgkin's disease, confirmed histologically. The culture of bone marrow aspiration showed *A. bovis*.

*A. bovis* was not found in the single bone marrow samples of two patients with clinically diagnosed and histologically confirmed Hodgkin's disease.

Six patients with various blood dyscrasias, requiring bone marrow studies, were used as controls. Their bone marrow was cultured as described, with negative results.

#### RESULTS AND SUMMARY

*A. bovis*, in pure culture, was isolated from the body fluids and tissues of four patients with proved cases of Hodgkin's disease, from one with a disease associated with brucellosis, from one diagnosed clinically as having abdominal granuloma, and from one with xanthoma tuberosum.

From this group, the organism was recovered in nine out of nine bone marrow aspirations, two of the twenty-four samples of blood, and from the spinal fluid, liver biopsy section, and corneal scrapings.

*A. bovis* was not found in the single bone marrow samples taken from two proved cases of Hodgkin's disease and from six patients with blood dyscrasias, or from more than 1,000 samples of blood and approximately 7,500 other specimens grown in the same fashion as described during this investigation.

Earliest and most consistent growth was seen in the thioglycollate broth incubated in a carbon dioxide atmosphere and in Tryptose phosphate broth grown under anaerobic conditions. Growth occurred at an average of six days in both media.

This work is being published to present the incidental finding of *A. bovis* in diseases other than actinomycosis. It is not the intention of the authors to indicate that *A. bovis* is the causative agent of any disease other than actinomycosis. The finding of this organism in the tissues and body fluids of patients who have granulomatous diseases is provocative. It is entirely possible that if other disease entities were investigated, similar findings might result.

Patients not having clinical actinomycosis may harbor *A. bovis* in their mouths, teeth decay, and mucous membranes, whence it is feasible that the organism, under favorable conditions may enter the blood stream. Since *A. bovis* has been found repeatedly over several months of observation in the same patients it does not seem to be merely a transient invader. The obvious disturbance of the reticuloendothelial system in granulomata may render a patient more susceptible to a chronic bacteremia with involvement of the tissues and body fluids.

Serologic as well as further bacteriologic studies are being carried out as material becomes available.

Grateful acknowledgment is made to Dr. N. F. Conant, of Duke University, and Major P. R. Carlquist and Major L. R. Kuhn, of the Army Medical School, for confirming the identity of the organisms.

## DEUTERIUM OXIDE AND THIOCYANATE SPACES IN PROTEIN DEPLETION

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PREVIOUSLY, the partition of body fluid between the extracellular and intracellular spaces has been attended by indirection and assumption<sup>1,2</sup> This type of approach has shown the importance of such data in understanding the distorted electrolyte and fluid balance in such clinical conditions as diabetic acidosis<sup>3</sup> and infantile diarrhea<sup>4</sup> The simultaneous determination of total body water and extracellular fluid volume theoretically gives the intracellular fluid volume by difference without the assumptions which have been necessary in the past

Total body water has been measured by desiccation in the human being<sup>5</sup> and values of 58 to 67.6 per cent of the body weight have been obtained The technical difficulties of such a procedure are easily appreciated and the method is of course worthless for clinical investigation The ideal method is to use a tracer substance which will be everywhere in equilibrium with body water Sulfanilamide was for a time used for this purpose, and it gives values of the correct order of magnitude<sup>6,7,8</sup> Urea<sup>9,10</sup> and thiourea<sup>11,12</sup> have been tried Apparently none of these three substances distribute themselves equally and exclusively in the body water Since it has been shown that in normal adults of many mammalian species fat is a diluent and water is a relatively constant portion of the lean body mass,<sup>13</sup> it has been suggested that an independent method for total body fat be established and water determined from this relationship The specific gravity method for total body fat has been used for this purpose<sup>14,15</sup> and results of reasonable magnitude have been obtained One of the best theoretical approaches to this problem, the simultaneous determination of body fat and water through differential solubilities of gases in body tissue,<sup>16</sup> has been neglected from the experimental point of view The best data on total body water in man comes from isotopic measurements Hevesy and Hofer<sup>17</sup> first used deuterium oxide in man Using oral administration, they got a value of 63 per cent of body weight Moore obtained a value of 72.5 per cent in a human subject<sup>18</sup> Pace<sup>19</sup> obtained a value of 64.7 per cent of body weight in a human subject, using tritium oxide We decided to use deuterium oxide as a tracer for total body water, although when T<sub>2</sub>O of higher activity becomes available and simpler techniques for its measurement are developed it may well provide a better method

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Though many methods are in vogue for measuring extracellular fluid volume none at present seems satisfactory. Thiocyanate is rapidly distributed, is easy to determine chemically, and has a slow rate of urinary excretion. On the other hand, it enters the red blood cells and is secreted by several digestive glands. Most studies with thiocyanate in normal men give values of from 20 to 28 per cent of the body weight<sup>20, 21</sup>. Sucrose has the advantage of not being an electrolyte, and therefore correction for Donnan effect is unnecessary. Sucrose is excreted so rapidly in the urine that an accurate measure of sucrose space is difficult. The sucrose space<sup>21</sup> appears to be 17 to 20 per cent of the body weight<sup>22</sup>. Radiosodium and thiocyanate spaces have been compared in man<sup>23</sup>. In normal men the sodium space (corrected for sodium which has penetrated bone) is 21.1 per cent of body weight and thiocyanate space, 23.5 per cent of body weight. In another study thiocyanate space was compared with radiosodium and radiochloride space<sup>21</sup> in the dog. Here the volumes of distribution of  $\text{CNS Na}^+$  and  $\text{Cl}^-$  were 36.20 and 25 per cent respectively of the body weight. Thus the dog may show dissociation between the values for extracellular space determined by thiocyanate space and by other techniques<sup>24</sup>. Thiocyanate, sucrose, and sulfate ion have approximately the same volume of distribution<sup>1</sup>. Bromide has been used as a measure of chloride space<sup>2</sup>. Further doubt has been cast on the thiocyanate space as a true representation of extracellular fluid volume since in malaria, spotted fever, and bacteremia<sup>18</sup> it may approach the value of total body water. In normal individuals, one might feel certain that at least the order of magnitude of the thiocyanate space was correct for extracellular fluid volume because of the concordance in volumes of distribution of the several substances cited; but, in the nephrectomized rabbit, thiocyanate distributes itself in a volume of 30 per cent of the body weight while inulin—a substance which is known not to enter the red cell, nor to be secreted by digestive glands, nor to suffer any binding to plasma protein—has a volume of 20 per cent<sup>2</sup>. Inulin might well be the ideal substance to measure extracellular fluid volume, but a method will have to be developed to circumvent its rapid urinary excretion. In spite of these difficulties in interpretation of thiocyanate space values we have routinely used them in this work although we are at present critically evaluating the thiocyanate space in patients showing abnormalities in water metabolism.

This study of human beings is divided into three parts: a study of two normal subjects, three cases of protein depletion, and a rather detailed study of the rehabilitation of two cases of protein depletion by the use of very high protein intakes, of the order of magnitude referred to as 'hyperproteinization' in this laboratory<sup>25</sup>.

Hereinafter the thiocyanate space will be designated  $\text{SCN S}$ , the per kilogram thiocyanate volume,  $\text{SCN S/W}$ , the total body water,  $\text{TBW}$ , and the per kilogram body water,  $\text{TBW/Wt}$ .

#### METHOD

$\text{SCN S}$ <sup>1</sup> nitrogen balance, and general metabolism techniques were carried out as described previously from this laboratory<sup>2</sup>.  $\text{TBW}$  was measured using

deuterium oxide as tracer. A carefully measured volume of 99.8 per cent of heavy water was delivered slowly from a burette attached to an intravenous needle and a quantity injected sufficient to bring the concentration of  $D_2O$  in the body fluid in the range of between 0.1 per cent and 0.2 per cent. Ten milliliter samples of blood were drawn at one, two, and three hours following injection of  $D_2O$ . If the patient had had previous injections of heavy water, an initial 10 ml sample of blood was necessary as a blank. The  $D_2O$  concentration reached equilibrium in less than an hour, confirming a similar observation of Moore<sup>18</sup>. The three samples of blood may thus be treated essentially as triplicates. The blood was transferred to 125 ml flasks equipped with ground-glass joints which were promptly stoppered. The blood was then frozen as a film in the inside surface of the bulb by rotating the flask slowly in a Dewar flask filled with crushed dry ice. No organic liquid was ever mixed with the dry ice to form a freezing mixture, and organic vapors in the laboratory were scrupulously avoided to prevent contamination of the water samples. The flasks were rapidly attached to a vacuum distillation apparatus such that the water which was lyophilized from the blood was condensed in a trap cooled by dry ice. This water was purified by a modification of the method of Keston and co-workers.<sup>30</sup> Both the samples and the standards were passed through an electric furnace in an atmosphere of liquid air oxygen in order not only to oxidize the organic impurities present, but also to adjust the  $O^{18}/O^{16}$  ratio from that of "water" oxygen to that of "air" oxygen.<sup>31</sup> The water was then distilled from 15 ml flasks attached by means of ground-glass joints to a small condenser. The samples were distilled after adding a few crystals of potassium permanganate and a tiny fragment of dry potassium hydroxide. Since at this stage the volume of the water is 5 ml, one need not fear deuterium exchange with the replaceable proton of the alkali. An arbitrary middle fraction was collected from this distillation in 10 ml glass stoppered weighing bottles. (This distillation is best not done on humid days.) The concentration of the deuterium was then determined by a modification of the falling drop method of Keston, using instead of the mercury filled capillary pipette which has hitherto been used by other workers, a constriction pipette of the type described by Levy.<sup>32</sup> This pipette has the advantages of great reproducibility, ease of construction, and freedom from the ever-present danger of air leaks which can occur with the mercury column pipette. The one-, two-, and three hour samples show an average deviation from the mean of 3 per cent. Times of fall, in the concentration range of 0.1 to 0.2 per cent,  $D_2O$ , agreed on an average of 0.2 second (or 0.002 per cent,  $D_2O$ ). Temperature was controlled to  $\pm 0.01^\circ C$ .

#### EXPERIMENTAL SUBJECTS

The normal subjects, H and T, were members of our own research team (Table I). The four depleted subjects, D, F, B, and C, were patients in the metabolic ward of the Third Surgical Service of the Bellevue Hospital. D and F had carcinoma of the tongue and granuloma of the sigmoid respectively. In these two only initial determinations were made. In both B and C, serial determinations were performed during different phases of repletion. Abstracts of the histories of these last two subjects appear below.

TABLE I TOTAL BODY WATER AND RELATED VALUES OF TWO NORMAL AND FOUR DEPLETED SUBJECTS

SUBJECT	WT (kg)	SCN S (LITERS)	SCN S LEAKAGE (CC)	TBW (LITERS)	TBW % WT	IW (TBW CVS) (LITERS)	TBS (WT TBW) (kg)	IW/ TBS
H	91	21.7	238	50.0	54.9	28.3	41	69
T	72.5	21.1	291	42.4	58.5	21.3	30.1	71
D	47.3	20.0	436	31.1	65.8	10.5	16.2	65
F	44.5	20.6	463	33.6	70.4	13.0	10.9	119
B 9/5	50.7	20.8	410	37.0	73.0	16.2	13.7	118
10/2	52.7	19.4	349	33.1	62.8	13.7	19.0	70
11/13	59.3	20.4	344	35.1	50.2	14.7	24.2	61
C 9/29	39.3	10.7	425	26.9	68.5	10.2	12.4	82
12/18	47.3	10.1	340	28.2	59.6	12.1	19.1	63
1/21	50.0	10.7	334	28.3	56.0	11.6	21.7	53
3/1	13.9	13.9	26.0	23.4	54.1	14.5	24.1	60

SCN S sodium thiocyanate space

TBW total body water

IW intracellular water

TBS total body solids

*Subject B*—A 39 year old colored man whose premorbid weight was 71.0 kilograms had swallowed live in a suicidal effort on April 6 1947. Prompt emergency treatment was given, but an esophageal stricture developed and on July 8 his weight was 53 kilograms, total plasma proteins were 6.25 Gm per cent Hgb 11 Gm per cent and RBC 3,950,000. On July 19 gastrostomy was done and the patient was hyperalimentated through the gastrostomy opening in an effort to restore the nutritional status. His gain in weight and strength was quite satisfactory as can be seen in Table I and on November 13 he weighed 59.3 kilograms, had improved greatly in strength and the plasma proteins were 6.8 Gm per cent, Hgb 13.8 Gm per cent and RBC 4,400,000. On Jan 15 1948 a transthoracic esophageal resection was done with closure of the gastrostomy.

*Subject C*—A 53 year old Chinese man whose premorbid weight had been 66.5 kilograms was admitted to Bellevue Hospital Psychiatric Service July 7 1947 after he made a suicidal effort at another hospital where he had undergone laparotomy for duodenal ulcer with bleeding. At that time Hgb was 4.7 Gm per cent RBC 1.17 million and 2+ ankle edema was present. On transfusion of blood and administration of protein hydrolysate the patient became rational and was up and about the ward on July 20. On August 19 an infradiaphragmatic vagotomy with gastroenterostomy was done and the patient was sent to the metabolic ward to be rehabilitated since his weight was 35.0 kilograms. D, F, B and C had lost 36, 38, 28, and 36 per cent of body weight.

## RESULTS

An example of the reproducibility of results obtainable with the method of determining TBW is seen on examination of the data obtained from the normal subjects H and T.

HOURS AFTER D<sub>2</sub>O1  
2  
3

## SUBJECT H

49.1  
51.3  
49.6  
50.0 L

## SUBJECT T

42.9  
43.0  
41.3  
42.42

H had a total body water of 55 per cent of his body weight. If the concept of lean body mass<sup>5</sup> is correct, and 73.2 per cent of this man's lean mass is water, then lean mass is 68.3 kg, and his fat content 22.7 kg, or 25 per cent of body weight. His moderate obesity explains his somewhat low water content.

The extracellular fluid volumes for H and T were 21.7 and 21.1 respectively. The ratios of thiocyanate space to weight (CNS/W) were 238 and 292 ml per kilogram. It will be noted that the thiocyanate space represents 43.4 and 50 per cent of total body water.

As may be seen in Table I, all of the depleted subjects showed expanded SCN-S at the initial test, the initial per kilogram values all being over 400 cc, and the ratio SCN-S/TBW being 66.3, 61.4, 56.2, and 62 respectively, while in the two normals the corresponding values were 50 and 42.4. This expansion of the SCN-S has been reported by other workers, among whom must be mentioned Lyons,<sup>33</sup> Mollison,<sup>34</sup> Walters and associates,<sup>35</sup> Henschel and associates,<sup>36</sup> and Co Tui and co-workers.<sup>28</sup>

The ratio TBW/Wt is also worth noting. In the two normal subjects this value was 0.550 and 0.585. In the four depleted subjects (D, F, B, and C) this ratio ranged from 0.655 to 0.730.

If the SCN-S were a faithful representative of the extracellular space, it would be possible to calculate the degree of hydration of the intracellular compartment. This could be achieved by obtaining two factors: (a) the total body solids, TBS, by subtracting the weight of the TBW from the body weight, and (b) the intracellular water, IW, which can be obtained by subtracting the extracellular volume from the TBW. The ratio IW/TBS would represent the hydration of the intracellular compartment, assuming that all but a negligible portion of the body solids were in the intracellular compartment. While fully aware that it may be premature at this point to assign any value to this ratio, one is nevertheless tempted to attempt some speculation in this direction. The IW/TBS ratios in the two normal subjects were 0.69 and 0.71. In three of the depleted subjects (F, B, and C), the corresponding values were 1.19, 1.18, and 0.82 respectively. In D it was 0.65. If these values were in fact correct, they would suggest an inconstant state of intracellular hydration in protein depletion. In view of the great variability of the plasma volume, body weight, and clinical edema demonstrated in protein depletion by Denz,<sup>37</sup> this possibility would be interesting to follow up in future studies.

TABLE II. PREMORBIDITY AND PRESENT TOTAL BODY SOLIDS OF DEPLETED SUBJECTS

SUBJECT	IPREMORBIDITY WT (KG)	WEIGHT LOSS (%)	TBS <sub>1</sub>	TBS	PER CENT TBS LOSS
D	73.9	36	29.56	16.2	48
F	71.8	38	28.76	10.9	62
B	70.4	28	28.16	13.7	51
C	61.4	36	24.56	12.4	49

TBS<sub>1</sub>, premorbidity TBS (estimated)

In Table II the premorbidity total body solids TBS<sub>1</sub> computed as 40 per cent of the weight of the four depleted subjects are set forth against their present total body solids, TBS calculated from the present body weight and the determined total body water. It will be seen that the percentage fall in body solids was in every case higher than the percentage fall in body weight and that in one subject, F, this fall was as high as 62 per cent. If this type of calculation



is valid, this means that 68 per cent of the subject's body substance has been lost. In this connection, it is significant that in the subjects B and C in whom serial determinations were made during repletion the values of TBS rose consistently as repletion progressed and that TBW/Wt, IW/TBS, and SCN S/W dropped.

It would also be of interest to gain an approximate knowledge of the state of total body fat both in depletion and during repletion. Since severely ill patients may not tolerate the procedure of total body fat determination by specific gravity method, one may make an approximation of this value by assuming a constant relationship between lean body mass and total body water in the normal individual as done by Paez and co-workers<sup>19</sup>.

One may assume that subject B was 'normal' on November 13. His lean body mass may then be computed since, according to this concept,  $0.732 \times \text{lean body mass} = \text{total body water}$ . This would give a lean body mass of 48 kg at this time or a total fat of 11.3 kg, which would amount to 19.1 per cent of the total body weight.

From the period September 5 to November 13 B retained about 1,100 Gm of N or 6.9 kg of protein. The total weight gain was 8.6 kg, and total body water decreased by 2 kilograms. Since the gain in total solids was therefore 10.6 kg, 3.7 kg of fat must have been formed during this period. From this gain of 3.7 kg in fat we can reason that on September 5 the total fat was 7.6 kg or 14.9 per cent of body weight at that time.

Our nitrogen retention data on subject C are not complete enough to warrant estimation of changes in fat and protein content.

Such calculations make one increasingly aware of the desirability of an independent method for measuring total body fat. During the process of repletion, the water content of the body expressed as a percentage of body weight is influenced by the loss of extracellular fluid, the retention of nitrogen, the presumptive retention of water which should occur with nitrogen retention to form muscle, and the gain in fat. These are factors which have been well evaluated in the crease analysis studies of Benditt and associates<sup>38</sup> in the rat, but which remain difficult problems in the human being.

In our work we were not able to observe the retention of water corresponding to nitrogen retention. In order to preserve osmotic equilibrium it is required that 3 Gm of water be returned for every gram of protein formed but actually it appears that in the process of depletion, protein may be lost at a faster rate than water, and it may well be that the partially ionized potassium compounds which have been postulated as occurring in intracellular water defend the osmotic integrity of the cell by further dissociation when protein is lost.

#### COMMENTS

This preliminary work is being reported because to our knowledge it is the first attempt of its kind in the study of the body compartments and constituents in protein depletion. It has already demonstrated that there is a profound

disturbance in both the body constituents and compartments in protein depletion, disturbances which have been assumed heretofore but have not been susceptible of direct measurement. The disturbance in water metabolism does not seem to be in the SCN-S alone, but seems also to extend to the intracellular compartment as well.

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## PARENTERAL NUTRITION

### VIII THE VASODEPRESSOR ACTIVITY OF SOYBEAN PHOSPHATIDE PREPARATIONS

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IN PREVIOUS reports from this laboratory the preparation and use of emulsions of fat for intravenous nutritional purposes have been described<sup>1,2</sup> Various emulsions containing soybean phosphatides as the stabilizer have been given successfully to the rat, dog, and rabbit. It was clearly shown in these studies that fat given in this manner is well utilized for energy. More recently, studies using fat labeled with C<sup>14</sup> have confirmed the utilization of fat in emulsions given intravenously.<sup>3,4</sup>

The only adverse physiologic effects which were evident when fat emulsions were given to dogs intravenously were occasional nausea, vomiting, and excessive salivation when the initial infusions were given too rapidly and a moderate normocytic anemia<sup>5</sup> if the fat infusions were continued for a period of two to three weeks or longer. The anemia rapidly disappeared, however, when the infusions were discontinued. Measurements of the blood pressure of dogs by an ordinary sphygmomanometer disclosed no significant alterations from normal. However, in a few preliminary observations in man following the intravenous injections of these fat emulsions, a fall in arterial blood pressure occasionally resulted. Before continuing with clinical studies, observations were undertaken in which the cat was used in order to obtain more information on this vasodepressor phenomenon. The experiments reported in this paper were designed to (1) determine the origin and character of the vasodepressor activity, (2) compare more rigorously the species variation with respect to the vasodepressor action, and (3) investigate possible methods of removing the vasodepressor substance.

#### EXPERIMENTAL

##### *Methods—*

1 Measurement of arterial blood pressure was done in the usual manner. The carotid artery of the anesthetized (Nembutal) animal was cannulated and by means of a citrate filled system was connected to a mercury manometer provided with a moving stylus. Recordings were made on a smoked paper drum attached to a kymograph. In many cases, concurrent pneumograph recordings were also taken.\*

2 Injection of the test substance was done as follows. A glass cannula was inserted into the femoral vein and by means of a narrow bore rubber tube was connected to a

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burette which contained either 0.9 per cent sodium chloride or 5 per cent dextrose solution. The test material was injected from a suitable syringe through a short No. 24 needle which was inserted as far as possible through the rubber tube close to the cannula. Immediately after the injection and before withdrawal of the needle 1 to 2 ml of the solution from the burette were allowed to run in as rapidly as possible. The syringe was then quickly withdrawn.

3. Emulsification of the test material usually was accomplished in one of the following ways: (a) stirring with a wire stirrer at 4000 rpm and 90° C for four to seven minutes; (b) blending in the Waring Blendor at high speed and at 70 to 90° C for one to five minutes; and (c) first blending as under (b) and then homogenizing in a high pressure homogenizer\* under nitrogen at 3000 to 4000 psi and 85 to 90° C for five to fifteen minutes. Where modifications of these methods were used the changes are indicated. Toxicity was relieved by the addition of saline or dextrose to give a final concentration of 0.9 and a per cent respectively. Adjustment of pH was accomplished with disodium phosphate.

4. The fat emulsifying properties of the various fractions of soybean phosphatides were determined in the following manner. One liter of an aqueous mixture containing 3 per cent of the phosphatide fraction 15 per cent coconut oil and 47 per cent dextrose was homogenized under high pressure by the technique previously described<sup>6</sup> but using the homogenizer referred to in the preceding paragraph. Disodium acid phosphate was used when necessary to adjust the pH. When the resulting emulsion was of fine particle size as judged by microscopic examination a sample was autoclaved and again examined for particle size.

*Origin and Character of the Vasodepressor Action and Variation Between Species*—Preliminary blood pressure measurements on the cat indicated that for this species a marked decrease in pressure occurred when even small quantities of an emulsion (Emulsion 35)<sup>1</sup> containing 3 per cent of a purified soybean phospholipid fraction were injected rapidly. This emulsion contained coconut oil 15 per cent fraction B(1'2) 3 per cent dextrose 47 per cent and water 80 per cent (weight/volume). It was readily apparent that a greatly decreased response resulted when a given dose was administered for the second time even though as much as thirty minutes elapsed between the two injections. To determine whether or not the soybean phosphatide stabilizer fraction B(F'2)<sup>1</sup> was responsible for the vasodepressor activity of the fat emulsion a 3 per cent emulsion of this material was prepared by high pressure homogenization made isotonic to rat serum with dextrose and autoclaved for fifteen minutes at 15 psi in a pressure bottle filled with nitrogen. Small quantities of this preparation were injected into a cat and the depressor response was found to be similar to that obtained when Emulsion 35 was administered.

Blood pressure studies were undertaken using the dog, rabbit and rat as test animals in addition to the cat. All blood pressure recordings were taken in a manner similar to that used for the cat with the exception of the rat. For this species it was necessary to use a capillary manometer and record the pressure changes visually. Neither the fat emulsion (Emulsion 35) nor the 3 per cent emulsion of the phosphatide fraction B(F'2) was effective in lowering the blood pressure of the rat rabbit or dog more than 15 mm even when large quantities were injected as rapidly as possible. Table I shows a typical response obtained with the cat, dog, rabbit and rat to the injection of a blended 6 per cent preparation of the phosphatide fraction B(1'2). It is readily apparent

that the cat is far more susceptible to the depressor activity and hence in all subsequent testing for vasodepressor activity the cat was used

TABLE I RESPONSE OF VARIOUS SPECIES TO THE VASODEPRESSOR PRESENT IN PHOSPHATIDE FRACTIONS MADE FROM COMMERCIAL SOYBEAN PHOSPHATIDES

SPECIES	BODY WEIGHT (GM)	MATERIAL INJECTED	VOLUME INJECTED (ML)	WEIGHT OF FRACTION INJECTED (MG/KG B W)	BLOOD PRESSURE RESPONSE	
					DECREASE (MM)	INCREASE (MM)
Rat	225	Blended 6% fraction B(F 2)	1.0	277	—	—
		Blended 6% fraction 70 f	2.0	554	14	—
Rabbit	3,100	Blended 6% fraction B(F 2)	2.0	39	—	4
		Blended 6% fraction 70 f	1.2	23	8	—
Dog	7,000	Blended 6% fraction B(F 2)	10.0	86	15	—
Cat	1,500	Blended 6% fraction B(F 2)	0.1	4	56	24
		Blended 1.2% fraction 70 f	0.1	0.8	46	—

Since it was evident that the phosphatide preparation B(F-2) contributed the vasodepressor activity, a number of the precursors of this fraction were investigated to determine whether the depressor was present originally in the soybeans or was introduced at some point during the process of manufacture. The following materials were tested in the cat for vasodepressor activity:

1 Freshly prepared soybean phosphatides obtained by extracting 25 kg quantities of ground dried soybeans for four successive three hour periods with 3 liter portions of boiling redistilled petroleum ether. The combined extracts were concentrated in vacuo to a volume of approximately 200 ml and 12 liters of anhydrous acetone were added and the mixture was allowed to stand overnight. After decantation of the clear supernatant liquid, the residue was dissolved in 40 to 50 ml of ether, filtered, and poured into 500 ml of acetone. The precipitate was filtered off after standing for three hours and was then dried in vacuo at room temperature. A 15 per cent emulsion of this material was prepared by the stirring technique, with dextrose present for toxicity, and examined for depressor activity.

2 An autoclaved portion of the 15 per cent emulsion of the freshly prepared soybean phosphatides.

3 Freshly prepared soybean phosphatides which had been allowed to stand in air for four days at room temperature and which were then emulsified at a 3 per cent concentration using the stirrer technique. Dextrose was added for toxicity.

4 Fat emulsion stabilized with freshly prepared soybean phosphatides and prepared by using the homogenization procedure described previously.<sup>6</sup> The emulsion contained 15 per cent refined corn oil, 2 per cent freshly prepared soybean phosphatides, 5 per cent dextrose, and water.

5 Commercial Liquid Lecithin\* emulsified at a 6 per cent concentration by the blender technique with dextrose present. This emulsion was not entirely stable and, therefore, only the portion which remained in true dispersion was used in the vasodepressor tests.

6 Asolectin (commercial soybean phosphatides) emulsified by the blender procedure at a 6 per cent concentration using dextrose for toxicity.

7 Emulsion 35<sup>1</sup>

8 Soybean phosphatide fraction B(F 2)<sup>1</sup> prepared in a 3 per cent emulsion by the high pressure homogenizer method using dextrose for toxicity.

9 Soybean phosphatide fraction B(F 2) prepared in a 6 per cent emulsion by the high pressure homogenizer method using dextrose for toxicity.

\*Liquid Lecithin, Robinson-Wagner Company, New York, N. Y.

†Asolectin was generously supplied by Dr. Albert Scharf of Associated Concentrates, Inc., Woodside, Long Island, N. Y.

TABLE II. EFFECT OF VARIOUS SOYBEAN PHOSPHATIDE PREPARATIONS ON THE BLOOD PRESSURE OF THE CAT

NO	MATERIAL	PER CENT OF PHOSPHATIDE FRACTION (WT/VOL.)	VOLUME INJECTED (ML.)	CAT		BLOOD PRESSURE RESPONSE*	
				NO	WT (KG)	DECREASE (MM)	INCREASE (MM)
1	Freshly prepared soybean phosphatides	15	0.8	1	0.97	—	10
2	No 1 autoclaved for 15 min at 15 psi	15	0.8	1	0.97	—	18
3	No 1 allowed to stand in air at room temperature for 4 days	30	0.4	1	0.97	24	—
4	No 1 + 15% corn oil as an emulsion	20	0.5	2	2.30	6	—
5	Liquid lecithin	60	0.5	3	0.67	10	—
6	Asolectin	60	0.1	4	1.60	28	—
7	Fraction B(F 2) + 15% coconut oil as an emulsion—Emulsion 35	30	0.4	5	2.00	50	—
8	Fraction B(F 2)	30	0.4	5	2.00	46	—
9	Fraction B(F 2)	60	1.0	8	1.60	77	—
	First injection					18	—
	Second injection (16 min after first injection)†					10	—
	Third injection (18 min after first injection)†						—

\*Refers only to the initial response

†In each case the blood pressure had returned to normal prior to the injection

These preparations were injected into cats as described earlier, and blood pressure recordings were taken. In addition, Preparation 9 was injected several successive times into a cat to test for tachyphylaxis. The results, together with pertinent data are given in Table II. It is seen that the depressor activity is primarily in the phosphatide preparations but is not present in freshly prepared material, even after autoclaving. Repeated injections of the phosphatide material result in greatly decreased vasodepressor responses. Fig 1 shows a typical response obtained with the cat to the repeated injection of a blended 6 per cent preparation of the phosphatide fraction B(F 2).

While the experiments already described were in progress, other experiments were concurrently undertaken for the purpose of fractionating the commercial soybean phosphatide preparation known as Asolectin and the phosphatide fraction prepared from it known as fraction B(F 2) from two stand points, namely, (1) to prepare if possible, a depressor free phosphatide fraction which would still be a suitable emulsifying agent and (2) to concentrate the vasodepressor substance(s) and determine some of its characteristics.

The initial studies were made on phosphatide fractions already on hand. These fractions had been prepared some months earlier in connection with fractionations of Asolectin designed to remove the material which had caused the granulomatous lesions reported in earlier work<sup>1</sup> and had been stored at  $-8^{\circ}\text{C}$  during the ensuing months. The preparation of fractions A(F 1), B(F 1), and C(F 1) have been reported<sup>1</sup>. Since the preparation of fractions D(F 1) and E(F 1) from B(F 1) was not given previously it will be described here.

## VASODEPRESSOR EFFECT OF SOYBEAN PHOSPHATIDE GIVEN IV

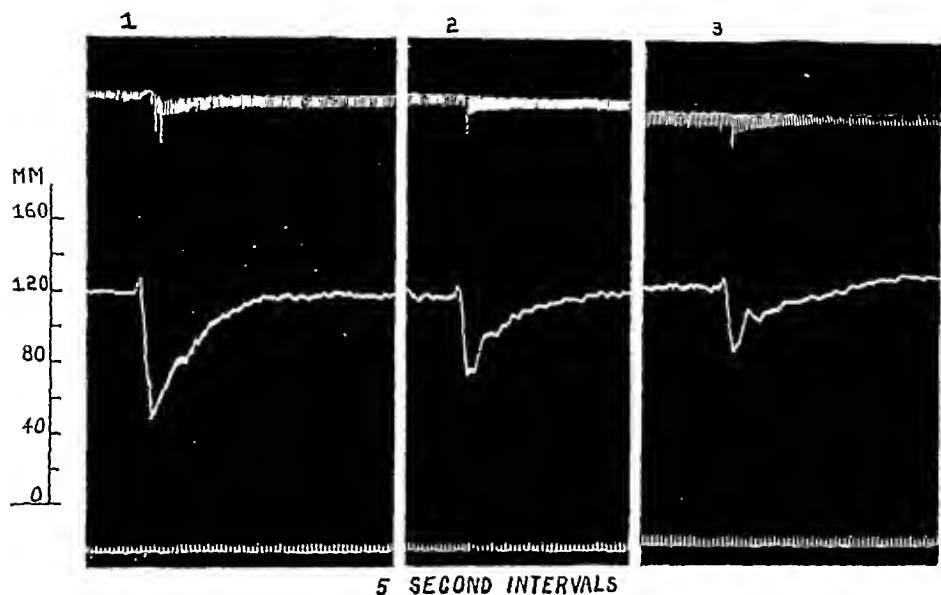


Fig 1—Response of blood pressure to repeated intravenous injections of 10 ml of a 6 per cent emulsion of soybean phosphatide fraction B(F-2) cat No 8 (16 kg). Time between injections 1 and 2 was sixteen minutes. Time between 2 and 3 was eighteen minutes.

*Preparation of D(F1) and E(F-1)* 205 Gm of fraction B(F1) were completely dissolved in 240 ml of dry chloroform and the solution was filtered. Three hundred sixty milliliters of 95 per cent ethanol were added slowly with shaking, and the light tan precipitate which formed was filtered off, dissolved in 100 ml of chloroform, and reprecipitated by the addition of 150 ml of 95 per cent alcohol. This material, fraction E(F1), was dried in vacuo and weighed 137 grams. The combined filtrates were concentrated to dryness in vacuo and yielded 6 Gm of fraction D(F1).

Each of the various fractions was blended, with dextrose present, and the vasodepressor activity was tested in the cat. Table III gives the results of these studies along with an evaluation of the emulsifying properties of the various fractions. The choline content of some of these fractions was determined by the method of Engel<sup>7</sup> and is included in the table since it serves as an index of the amount of lecithins present. Lipositol prepared according to the procedure of Woolley<sup>8</sup> was also included in these studies, and it was found to be no more active than the lipositol-low, alcohol-insoluble fractions.

The results obtained with all of the foregoing preparations indicated that the depressor substance was probably a degradation product of one of the phosphatides found in soybeans. Although the almost complete lack of solubility of the depressor substance in alcohol negated such compounds as free choline, ethanolamine, and many of their simple derivatives, some of these compounds were tested in the cat and the resulting effect upon blood pressure was compared with that of the unknown depressor. The character of the curve

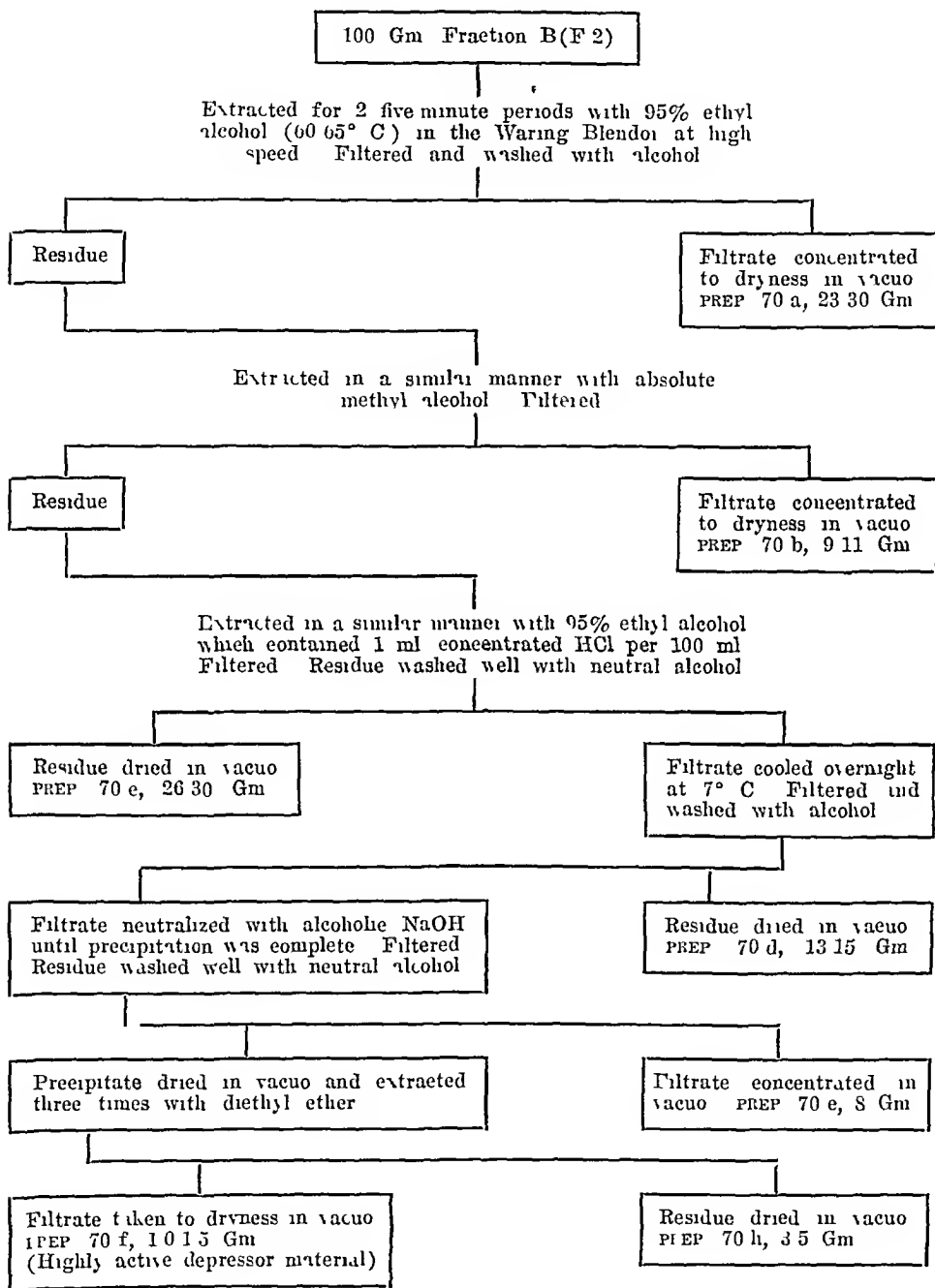


TABLE III EFFECT OF VARIOUS FRACTIONS MADE FROM COMMERCIAL SOYBEAN PHOSPHATIDES (ISOJECTIN) ON THE BLOOD PRESSURE OF THE CAT

NO	FRACTION	RELATIVE EMULSIFYING PROPERTIES*	CHOLINE CONTENT	CONCENTRATION PER CENT (WT/VOL)	VOLUME INJECTED (ML)	CAT			BLOOD PRESSURE ELUTIONSE	
						NO	WT (KG)	DECREASE (MM)	INCREASE (MM)	
1	A(F1)	Poor	—	30	10	7	—	—	6	
2	B(F1)	Fair	076	30	04	7	—	6	—	
3	C(F1)	Very good	105	30	10	7	22	—	4	
4	D(F1)	Fair	185	30	04	7	22	—	—	
5	E(F1)	Fair	004	30	02	7	22	54	—	
6	D(F2)	Good	—	30	03	6	15	56	—	
7	Lipostol	Poor	—	30	10	7	22	60	—	
8	Lipostol free residue	Fair	—	30	10	7	22	55	—	
9	70 f	—	—	12	01	6	15	46	—	

\*Based on the rating of good for fraction B(F-)

## CHAPTER I FRACTIONATION OF SOYBEAN PHOSPHATIDE FRACTION B(F2) SCHMIDT 74

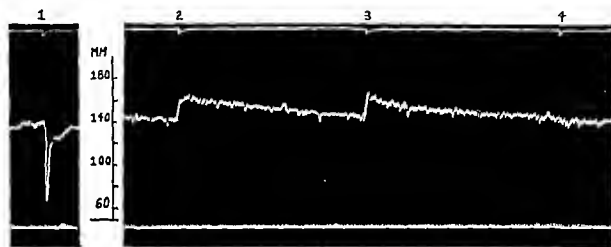


of the blood pressure response was not similar to that of the material in the soybean phosphatide fractions, and the extreme tachyphylaxis which resulted on repeated injection of phosphatides was also absent.

The following preparations also were made and tested for depressor activity

1 A dialysate of the fraction B(F 2) prepared by dialyzing a 6 per cent emulsion of this fraction against distilled water through a thin collodion membrane and concentrating the dialysate to the volume of the original emulsion. This material was inactive, whereas the residue was still completely active.

2 Ten milliliters of Emulsion 35 placed in a 15 ml test tube and to which 0.5 Gm of sodium chloride was added. The tube was heated at 70°C for five minutes and then centrifuged while hot. The aqueous layer was tested for activity and was completely inactive.



SIX SECOND INTERVALS

Fig. 2—Effect on blood pressure of route of administration of soybean phosphatide fraction 70 f cat No. 9 (2.3 kg)

- 1 0.1 ml 2.5 per cent 70 f intravenously
- 2 0 ml 2.5 per cent 70 f intraperitoneally
- 3 2.0 ml 2.5 per cent 70 f intraperitoneally
- 4 1.0 ml 2.5 per cent 70 f subcutaneously

Further fractionations of the preparation B(F 2) were carried out by means of solubilities in various solvents such as ethyl alcohol, acetone, chloroform, and mixtures of these solvents but none was effective in concentrating the depressor to a sufficient degree. Since the activity in the earlier fractionations followed those fractions high in inorganic constituents, separations were undertaken with acidic solvents. Acidic ethyl alcohol proved to be best for this purpose, and although many different fractionation schemes were carried out only one of the most successful is described in Chart I. This fractionation of B(F 2) yields a material referred to as 70 f which is a highly active depressor substance. The results of the tests for vasodepressor activity using fraction 70 f are given in Tables I and III. The marked susceptibility of the cat to the vaso depressor activity is well shown in the comparative data in Table I.

*Effect of Route of Administration (Other Than Intravenous) on Depressor Activity of Fraction 70 f*—To determine the effect of the route of administration on depressor activity, a 2.5 per cent emulsion of preparation 70 f was injected intraperitoneally into an anesthetized cat. Fig. 2 shows the results of a typical experiment, and it is apparent that instead of causing a decrease in blood pressure, a pressor response resulted. Oral administration of this preparation as well as intramuscular and subcutaneous administration was without effect. Hence the depressor effect of the phosphatide fraction is only evident when the material is given intravascularly.

*Effect of the Depressor Substance on Duodenal Muscle*—To determine whether or not the depressor material had an effect on smooth muscle contractions, a strip of rat duodenal muscle was connected to a lever in the usual manner and was immersed in oxygenated mammalian Ringer's solution. At intervals 0.4 ml of a 2.5 per cent emulsion of fraction 70-f was added, in each case the Ringer's solution was changed before the addition. Five per cent glucose solution was used as the control. The results of this experiment are

#### EFFECT OF DEPRESSOR MATERIAL ON SMOOTH MUSCLE CONTRACTION

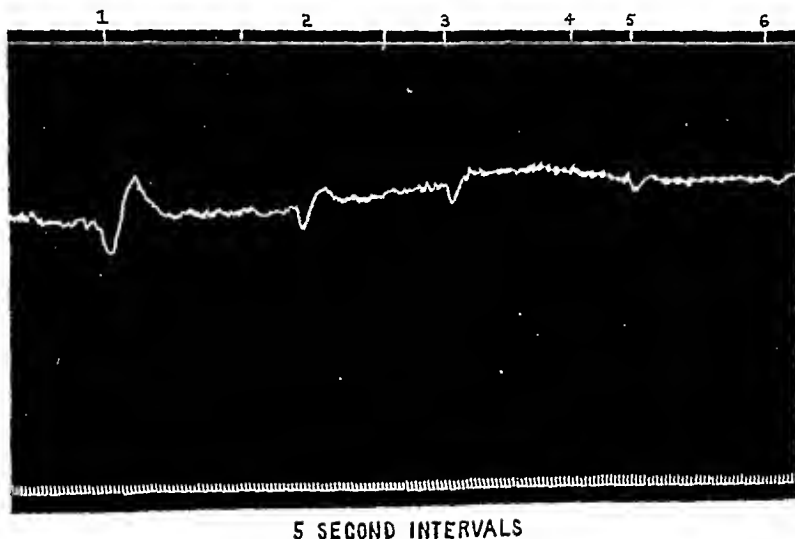


Fig. 3—Contraction of rat duodenal muscle to successive stimulation with 0.4 ml of an emulsion of 2.5 per cent of soybean phosphatide fraction 70-f. Volume of Ringer solution was 200 milliliters,  $\frac{1}{2}$  was 5 per cent dextrose solution. Time lapses between 2 and 3, 3 and 4, and 5 and 6 were 5, 6, and 5 minutes respectively.

given in Fig. 3 and it is observed that the depressor material gives rise to a marked contraction of the muscle and that the contraction decreases with repeated addition of fraction 70-f.

#### DISCUSSION

The soybean phosphatide fraction B(F-2) described previously<sup>1</sup> for use as a stabilizer in fat emulsions for intravenous nutritional purposes has been found to possess a marked vasodepressor activity when injected into the cat. This activity was present in the commercial soybean phosphatide preparation from which this fraction was obtained. Since freshly prepared soybean phosphatides had little such activity, vasodepressor substance probably is formed sometime during or after the process of manufacture by oxidative or degradative changes. This view is supported by the fact that freshly prepared phosphatides became active depressants when allowed to stand at room temperature in contact with air.

The great species difference with respect to this vasodepressor activity was of interest. In the cat, 4 mg of fraction B(F-2) per kilogram of body weight caused a decrease in arterial blood pressure of 56 millimeters. Little or no

effect was obtained however, when 277 39 and 86 mg quantities per kilogram of body weight were given to the rat rabbit and dog, respectively. This difference is important in work concerning intravenous fat emulsions because the dog and rat are the animals usually used for testing such preparations. From clinical studies it appears that man is more sensitive to the vasodepressor than these three species but less so than the cat. It is obvious that it is desirable to test fat emulsions intended for clinical use in several species including the cat.

Several interesting characteristics of the pattern of the blood pressure response to the various phosphatide fractions have been observed. One of these was the step like manner in which the blood pressure returned to normal. This ranged all the way from a slight pause or dip to a drop back to the original low. In the latter case the return proceeded without any sign of another stepwise recant. Such a step phenomenon is not characteristic of the response to such compounds as acetylcholine histamine etc. Another difference between the soybean depressant and the other depressant materials tested was the relatively great tachyphylaxis that occurred following the initial response when an original drop of more than 10 to 20 mm had occurred. This tachyphylaxis made the exact quantitative evaluation of the potency of various preparations difficult but a fair comparison was still possible by allowing a suitable time lapse between injections and by using a standard injection of an emulsion of the fraction B(F 2) as an index of the tachyphylaxis.

The vasodepressor substance was not one of the known phosphatides or their simple degradation products such as free ethanolamine. It was nondialyzable through collodion and insoluble in water alcohol and aqueous acid but soluble in acidified alcohol. The use of the latter solvent especially when hot may have resulted in various extraneous cleavages but no apparent increase in total original depressor potency occurred through its use. Perhaps the acid functioned by removing moisture ions from the depressor and thereby rendered it or its hydrochloride alcohol insoluble. Partial neutralization of the acidic solution caused the precipitation of the depressor along with salts. Ether extraction removed it from the latter and yielded a light yellow nitrogenous material.

The effect of the depressor material in causing a contraction of duodenal muscle suggests that the material could act directly on the blood vessel wall. It is of interest to note that a decreased stimulation of the muscle strip resulted on repeated application of the depressor. The sustained elevation in blood pressure which occurred when the phosphatide fraction 70 f was given intraperitoneally may have resulted because of stimulation of intestinal contraction.

Because of the vasodepressor effect of phosphatide fraction B(F 2) Emulsion 35<sup>1</sup> is not considered suitable for clinical use though it is adequate for experimental studies in the dog rat or rabbit. Although phosphatide fractions low in depressor activity could be prepared these were either poor emulsifying agents or if they were suitable as an emulsifying agent they underwent changes on autoclaving that gave rise to toxic effects. Fraction C(F 1) (Table III) is a good example of a phosphatide fraction with no depressor activity good emulsifying properties but one which is unsatisfactory following autoclaving. In general it appears that the more a phosphatide preparation is purified

the less stable it becomes to autoclaving. Fraction B(F-2) still remains the most suitable of the soybean phosphatide fractions for stabilizing fat emulsions for intravenous use. Only approximately half of fraction B(F-2) possesses good emulsifying properties, but the remainder of the preparation appears to provide stability to the fraction during autoclaving.

The question arises as to whether fraction B(F-2) can be used in a concentration of less than 3 per cent (as it is used in Emulsion 35) and stabilize an emulsion, yet not be present in such an amount that a depressor effect will be obtained. Emulsions containing 15 per cent fat, identical in composition to Emulsion 35 except that fraction B(F-2) has been reduced from 3 per cent to 0.5 per cent, have been prepared, and they do not affect the blood pressure of the cat. However, stability on standing and when added to plasma is not as great as desired. Further studies utilizing a costabilizer in addition to 0.5 per cent of fraction B(F-2) are reported in the following paper.

#### SUMMARY

1 A soybean phosphatide preparation, B(F-2), prepared for use as a stabilizer for fat emulsions for intravenous nutrition has been found to possess vasodepressor activity in man and the cat, little such activity in the dog, and none in the rat or rabbit.

2 The depressor material is not present in phosphatide fractions freshly prepared but develops, on standing, on contact with air. It does not appear to be acetylcholine, choline, ethanolamine, or any simple derivatives of these compounds. The depressor material has been concentrated considerably.

3 Fractions of phosphatide low in depressor activity have been prepared, but are either undesirable emulsifying agents or are adversely affected by autoclaving.

We wish to express appreciation to the following companies which have supplied us generously with various materials used in this research: The Upjohn Company, Kalamazoo, Mich.; Merck and Company, Inc., Rahway, N. J.; Games Division of General Foods Corporation, Hoboken, N. J.; Wilson Laboratories, Chicago, Ill.; Sheffield Farms Co., Inc., New York, N. Y.; Corn Industries Research Foundation, New York, N. Y.; and Anheuser-Busch Co., St. Louis, Mo.

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## PARENTERAL NUTRITION

### IX FAT EMULSIONS FOR INTRAVENOUS NUTRITION IN MAN

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IN THE preceding paper<sup>1</sup> it was shown that the soybean phosphatide fraction known as B(F 2) which was used to stabilize emulsions caused a vasodepressor response in certain species particularly man and the cat, when used at a concentration of 3 per cent of the total emulsion. Although various depressor free fractions could be prepared from soybean phosphatides, they were either poor emulsifying agents or were adversely affected by autoclaving. Phosphatide fraction B(F 2)<sup>2</sup> remains the most useful stabilizing agent used in this laboratory for fat emulsions intended for intravenous nutrition, but if it is so used it must be present in concentrations less than 1 per cent if the emulsions are ultimately intended for clinical use in man. The purpose of this paper is to report observations on fat emulsions stabilized with 0.5 to 1 per cent of B(F 2) with and without the use of a costabilizer, and preliminary studies on the use in man of one of these fat emulsions given intravenously.

### EXPERIMENTAL

All emulsions were prepared under nitrogen by the high pressure homogenization technique previously described using a pressure of 3500 to 4000 psi and a temperature of 80 to 92° C for five to ten minutes. Sterilization was accomplished by autoclaving the preparations in tightly capped nitrogen filled bottles for fifteen minutes at 15 psi. The size of the fat particles was ascertained by microscopic examination, and no emulsion was considered satisfactory from the viewpoint of particle size unless almost all of the particles were below 1  $\mu$  in size and no particles were observed larger than 4 microns.

The stabilizers used were soybean phosphatide fraction B(F 2) and Demal 14\* either singly or in combination. Coconut or corn oil in a final concentration of 15 per cent was used throughout. The composition and evaluation of these emulsions are given in Table I and it is seen that Emulsions 40 and 41 possessed the most desirable properties, i.e., very small particle size and no depressor effect in the cat or any other adverse physiologic effect following injection. Emulsions 40 and 41 contained 0.5 per cent phosphatide fraction B(F 2) as stabilizer and 1 per cent Demal 14 as costabilizer. Emulsion 40 was made with corn oil and Emulsion 41 with coconut oil. Since these two emulsions appeared to be most promising, only the experiments concerned with them will be reported.

*Animal Studies*—Six albino male rats weighing approximately 150 grams were injected daily with Emulsion 40 at a level of 2.0 ml. per 100 grams body weight. Injections were made through a tail vein while the animal was under

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TABLE 1 COMPARATIVE STUDIES ON 15 PER CENT FAT EMULSIONS STABILIZED WITH PHOSPHATIDE FRACTION B(F 2) AND D(MAY 14)

EMULSION*	STABILIZER CONCENTRATION		RELATIVE PARTICLE SIZE	PHYSIOLOGIC EFFECTS ON INJECTION†				REMARKS
	FRACTION B(F 2) (%)	D(MAY 14) (%)		CAT (5 ML)	RAT (5 ML)	DOG (30 ML)	HABBIT (10 ML)	
36	1.0	--	Very good	Visodepressor	None	--	--	Not used further because of moderate depressor action in the cat
37	0.5	--	Good	None	None	--	--	Stability in plasma considered border line Not used further
38	--	1.0	Fair	None	None	--	--	Particle size considered to be too great for continuous use
39	1.0	1.0	Very good	Visodepressor	None	--	--	Not used further because of moderate depressor action in the cat
40	0.5	1.0	Very good	None	None	None	None	Satisfactory—used in studies reported in this paper
41	0.5	1.0	Very good	None	None	None	None	Satisfactory—used in studies reported in this paper

\* All emulsions contained coconut oil 15 per cent, dextrose 4.3 per cent and water to make 100 ml with the exception of Emulsion 40 in which corn oil at 15 per cent was used as the source of fat

† Response following single injections. Amounts in parentheses are maximum volumes used per kilogram of body weight



light ether anesthesia. Twelve to fourteen daily injections were given and one week after the last injection the animals were autopsied. No gross changes were in evidence and histologic sections revealed no abnormalities. During the course of the injections a decrease in hemoglobin from an average of 14.5 Gm per 100 ml to 12.0 Gm per 100 ml occurred but a return to the normal values resulted during the postinjection period. Weight gain during the injection period averaged 2.4 Gm per day.

A similar experiment was performed with weanling rats and the results obtained substantiated those of the previous study.

Three adult female dogs weighing approximately 9 kilograms each were fed ad libitum a diet which consisted of 78 per cent Games Dog Meal, 2 per cent liver powder (Wilson 1920), 10 per cent brewers' yeast, and 10 per cent crude casin. All dogs were weighed and infused daily and hemoglobin, hematocrit, cell counts and liver function tests were done at weekly intervals.

Infusions were begun with Emulsion 40 containing 15 per cent corn oil. This emulsion was well tolerated by the animals in amounts of 3 Gm of fat per kilogram body weight per day even at infusion rates up to 10 ml per minute, and no vomiting resulted even during the initial infusions. After an average of ten days however a distinct anemia was observed. In two of the dogs, Emulsion 40 was replaced with Emulsion 41 identical in composition except that 15 per cent coconut oil was the source of fat. Emulsion 41 was also well tolerated, and quite unexpectedly the anemia of these two dogs (Dogs 1 and 2) disappeared almost completely, although infusions were continued for many days. The data for each dog are given in Table II and may be summarized as follows:

Dog 1 received daily infusions of fat for a period of eleven weeks. During the first five weeks the preparation was a corn oil emulsion (Emulsion 40) given in a dosage of 3 Gm of fat per kilogram per day and at rates varying from 5 to 10 ml per minute. The hemoglobin decreased from 15.4 Gm to 12.3 Gm per 100 ml and the hematocrit from 43.6 per cent to 30.5 per cent. At this time the animal's hemoglobin level was purposely reduced to slightly below 9 Gm/100 ml by bleeding. A marked reticulocytosis followed which demonstrated that the anemia produced by hemorrhage stimulated a reticulocyte response even though the animal had received daily infusions of fat for more than a month. At this time the coconut oil emulsion (Emulsion 41) was substituted and used during the last six weeks of the infusion period. It was given in increasing doses of 3, 4, 5, and 6 Gm per kilogram per day as indicated in Table II. There was a definite and rapid rise in hemoglobin, hematocrit and erythrocyte values during the last five weeks of infusion with the coconut oil emulsion despite the fact that the coconut oil was finally given at twice the level that the corn oil emulsion had been infused. Food consumption decreased as the quantity of fat infused was increased. The animal was sacrificed two weeks after the last fat infusion, autopsy disclosed no gross abnormalities and microscopic examination of tissue sections showed no abnormalities.

Dog 2 received twelve daily infusions of the corn oil emulsion (Emulsion 40) in the amount of 6 Gm of fat per kilogram of body weight per day and at a rate of 4 to 8 ml per minute. By this time a definite drop in hemoglobin and hematocrit had appeared. The fat infusions were stopped for four weeks by which time the blood picture had improved. Daily infusions with the coconut oil emulsion (Emulsion 41) were then started at the same rate and amount as had been used previously for the corn oil emulsion. While the hemoglobin and hematocrit again decreased during the next two weeks of infusion they began



DOO 3

WEEKS OF EXPERIMENT	1	3	5	7	8	9	10	11	12	13	14	16
15% FAT EMULSION GIVEN 1 V AS INDICATED BY BAR--FLOUR ON BAR GIVES GM OF FAT/KG/DAY			3		45							
Hemoglobin (gm/100 cc)	1687		1499	1039		1142	1140		1502			
Hematocrit (%)	49.0		41.1	29.0		31.0	33.0		46.1			
Erythrocytes (mil/c mm.)	790		626	447			627		600			
MCV ( $\mu^3$ )	62		65.6	65			52.7		70.8			
MCH ( $\gamma\gamma$ )	21.4		24	23			21.4		25.0			
MCHC (%)	34.4		37	36.3		36.8	40.1		32.6			
Leucocytes (thous/c mm.)	223		169	244			465		170			
Bronsulfalen ( $\gamma$ /c.c. at 8 mm)			18.2	18.8			18.8		10.4			
Body wt (kg)	835	922	930	1015	96	870	963		90			

to return to normal during the third and last week of infusion. Dog 2 was sacrificed two weeks after the last infusion and post mortem examination revealed no significant gross or microscopic changes from the normal.

Dog 3 received twelve daily infusions of the corn oil emulsion (Emulsion 40) at a level of 5 Gm of fat per kilogram of body weight per day and at rates varying from 5 to 10 ml per minute. These infusions were given, as it happened, during the last days of pregnancy. On the twelfth day, shortly after the infusion had been given, six healthy puppies were born. Fat infusions were continued for another twelve days but at an increased level of 45 Gm per kilogram body weight, and despite the extra load imposed by nursing, the hemoglobin and hematocrit values returned rapidly toward normal. The data are given in Table II. Four weeks later this dog was in excellent health. No autopsy was performed.

A supplementary study was done with rats to determine whether a difference between coconut and corn oil could be shown in this species with respect to the development of anemia. Two groups of ten rats each were injected daily for twenty-one days. One group received the corn oil Emulsion 40 and the other the coconut oil Emulsion 41. Hemoglobin determinations were done at three- to four-day intervals. Both groups developed a moderate anemia and no significant difference between the two emulsions was obtained.

*Clinical Trials With Emulsion 11*—As a result of the preceding animal studies, it was concluded that either Emulsion 40 or 41 in which the stabilizing agents were 0.5 per cent B(F-2) and 1 per cent Demal-14 were adequate for a careful clinical trial. They could be sterilized by autoclaving, were of small particle size, stable in contact with blood, did not affect blood pressure, and, except for a moderate and transient anemia, produced no physiologic or pathologic abnormalities after repeated daily infusions for periods of several weeks. The initial clinical trials were made with the intention of determining the immediate reactions of the patients and their tolerance to the rate of administration. Balance studies to test utilization and the contribution of infused fat to energy requirements were not attempted in these preliminary, short-term studies. The first three patients selected represented clinical problems of a hopeless nature, but which might at the same time derive some benefit from the supplementary calories provided.

*Patient 1* This patient was a 75 year old woman who entered the hospital in 1937. A history was obtained of cardiovascular disease of several years' duration with symptoms of decompensation and signs of auricular fibrillation of three months' duration. Examination revealed a right hemiplegia. The patient remained semicomatose and unable to swallow. Tube feedings used initially were discontinued because of vomiting and the danger of aspiration. Although the patient could be kept hydrated with clysis, caloric insufficiency produced a weight loss of 33 pounds in the first six weeks of hospitalization and the patient became emaciated. On the forty-second hospital day, the patient was given 300 ml of Emulsion 41 (460 calories) in a period of 110 minutes. The infusion was made through a No. 20 needle. The equipment was arranged so that either the fat emulsion or a solution of 5 per cent glucose in water could be infused by gravity through separate Murphy drip bulbs by adjustment of the valve of a three way stopcock attached to the needle. Control determinations of temperature, pulse, respiration, and blood pressure were obtained at three ten minute intervals while glucose was being infused, then the stopcock was turned so as to substitute the emulsion. When infusing conscious patients, this arrangement allowed arbitrary choice of solutions without the patient's knowledge in order to better evaluate subjective symptoms.

# RESPONSE OF PATIENT 1 DURING INFUSION OF 300 ML OF FAT EMULSION (NO 41) INTRAVENOUSLY

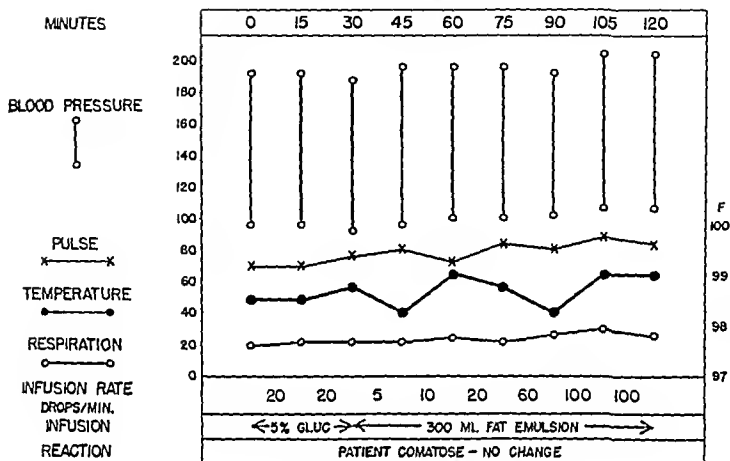
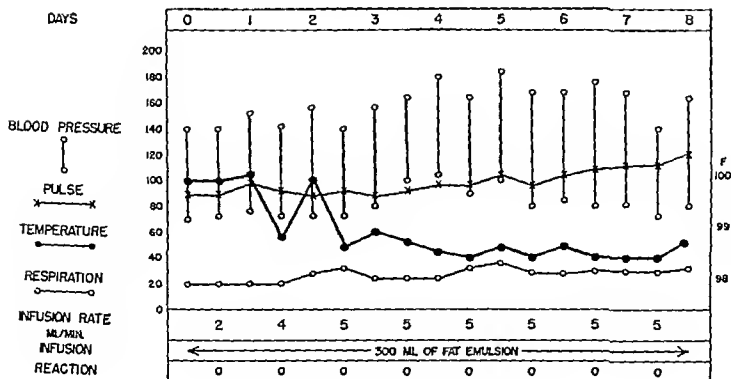


Fig 1

# RESPONSE OF PATIENT 2 DURING 8 SUCCESSIVE DAYS OF INTRAVENOUS INFUSIONS WITH 300 ML OF FAT EMULSION (NO 41) DAILY



Fig

Fig 1 illustrates the response of Patient 1 to this infusion and it is seen that no abnormal reaction was observed. There was no further change in the clinical status of this patient. She was transferred to a nursing home two days later.

*Patient 2* This patient was a 78 year old woman admitted in a semicomatose state with a history of two years of mild "strokes" and a gradual loss of consciousness twenty four hours prior to admission. Physical examination showed an obese, plethoric woman, with enlarged heart and liver, pitting, ankle edema, and a right hemiplegia. The clinical diagnosis was multiple cerebrovascular thrombosis and generalized arteriosclerosis. The patient lost weight rapidly on a regimen of parenteral glucose, protein hydrolysate, and saline.

On the ninth hospital day the patient was recovering from a mild infection and had a temperature of 100° F. On this day 300 ml of Emulsion 11 were given without reaction. This amount was given on each of the succeeding seven days, amounting to 2,100 ml in all. Fig 2 illustrates the response of Patient 2 to these infusions, and it is seen that there was no abnormal reaction. During this period there was some improvement clinically of the patient's sensorium.

Since previous animal studies had indicated that continuous infusions of emulsions led to a mild anemia, this patient was followed with hematologic studies. During the eight days of infusion the patient's hemoglobin fell from 15.3 Gm to 12.0 Gm per cent. The hematocrit fell from 51 per cent to 42 per cent. This reduction of hemoglobin and hematocrit was undoubtedly due in part to the fat infusions.

Eleven days after the last infusion this patient expired and an autopsy was performed. At this time the patient was described as poorly nourished. The spleen, liver, and lungs were described as grossly normal. Histologically, the lungs revealed occasional small areas of atelectasis with a few infiltrating lymphocytes. The liver cells showed a fine cytoplasmic vacuolization similar to the changes of starvation and protein depletion. No granulomatosis or giant cell containing lesions were seen reminiscent of the lesions previously described in animals after infusions of some of our earlier emulsions 3, 4.

A fat tolerance curve done after the seventh infusion in this patient by the turbidimetric method previously described<sup>5</sup> indicated the same prompt fall in concentration of the visible fat particles in the plasma as previously reported for rats and dogs.

*Patient 3* This patient was a 24 year old man in the terminal stage of glomerular nephritis. There was a five year history of proteinuria. Nine months previously edema had appeared, and for the prior two months the blood urea nitrogen and blood pressure had been very high, with several convulsions before entry. The patient was treated periodically in the hospital with an artificial kidney and maintained on a low protein intake. It was felt that a source of nonprotein calories in high concentration might lessen the renal burden. On the sixth hospital day, this patient was given 600 ml of fat Emulsion 41 (920 calories) in 120 minutes without reaction. The emulsion used was a new batch which had been assayed for sterility, pyrogenicity, and infusion reactions in rats, dogs, and cats and found to be satisfactory in all respects. The patient was alert and cooperative on this day, having been treated the previous day with the artificial kidney. He experienced no subjective reactions and could not distinguish whether he was being infused with 5 per cent glucose or the fat emulsion. On the following day another 600 ml of Emulsion 41 were given at a maximum rate of 5 ml per minute without reaction. General clinical improvement was readily apparent, though how much of this was due to the treatment with the artificial kidney two days previously and how much due to the fat emulsion could not be ascertained. On the next day the patient was given a third infusion of 600 ml of Emulsion 41 and at the end of the infusion complained of chilliness. Two hours later the oral temperature had risen to 102° F. Since the patient had been on the artificial kidney that day and since four previous bottles of the same batch of emulsion had not produced pyrogenic reactions in this patient, the fat emulsion was not considered the cause of the temperature rise. The response of this patient during the three days of fat infusion is given in Fig 3.

This patient's clinical condition rapidly grew worse in the ensuing days and on the thirteenth hospital day he expired after a uremic convulsion. An autopsy was performed.

RESPONSE OF PATIENT 3 DURING 3 SUCCESSIVE DAYS  
OF INTRAVENOUS INFUSIONS WITH 600 ML OF FAT EMULSION (NQ 41) DAILY

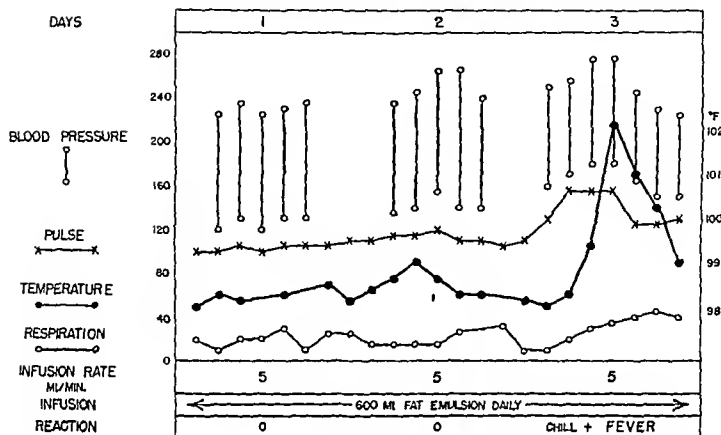


Fig 3

The visceral organs were essentially normal grossly and there was no histologic evidence of lesions attributable to the infused fat emulsion.

**Patient 4** This patient was an alert and intelligent 37 year old civil engineer who entered the hospital with a complaint of swelling of the ankles. There was a five year history of renal disease with a left monoplegia of one year's duration. The blood pressure was  $\frac{230}{150}$  with an enlarged heart and liver and extensive retinitis. This patient was placed on a low protein diet and treated with parenteral low salt albumin. With his complete and effective cooperation a trial of intravenous fat emulsion was made.

On the twelfth hospital day 600 ml of Emulsion 41 were given at a rate of 5 ml per minute without reaction. The patient stated that he experienced no unusual symptoms at the site of injection or elsewhere. There was no unusual taste sensation experienced although he stated that for two or three hours after the infusion he experienced a sensation of languor and epigastric fullness much like the satiation following a large meal. There was no nausea.

The following day another 600 ml of fat emulsion were given at a rate of 5 ml per minute. At the end of the infusion the patient had a sudden, mild shaking chill and in the next three hours the temperature rose to 100.8°F. The blood pressure was not changed. Emulsion from the infusion set was cultured and found to be sterile. The following day a third infusion of 600 ml of emulsion was given again. The last 300 ml were given at a rate of 12 ml per minute. There was no reaction subjectively or of the temperature or blood pressure. Hematologic studies on this patient revealed a fall of the hematocrit from 35 to 33 per cent and of the hemoglobin from 12.2 Gm to 11.5 Gm over the period of fat infusion. This patient was discharged with his clinical condition essentially unchanged.

**Patient 5** This patient was a 40 year old housewife with a complaint of shortness of breath and swelling of the ankles. There was a five year history of chronic pyelonephritis.

RESPONSE OF PATIENT 5 DURING INFUSION OF  
300 ML OF FAT EMULSION (NO 41) INTRAVENOUSLY

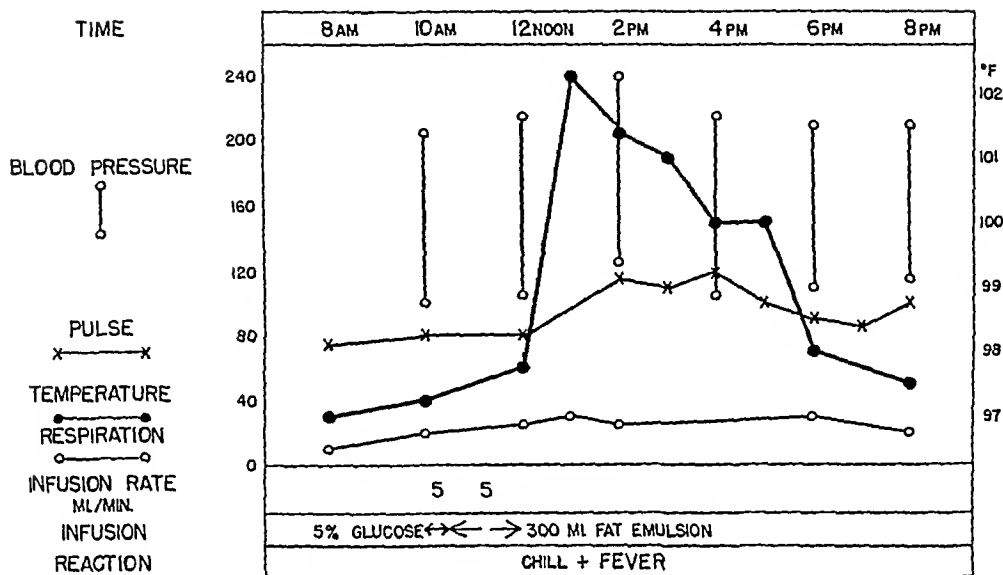


Fig 1

with mild hypertension and retinitis. On a low protein, low salt diet, she had done well although there was evidence of increasing azotemia.

The patient was given 300 ml of fat emulsion by the usual method with an infusion rate of 5 ml per minute. At the close of the infusion she experienced a shivering chill. The temperature rose to 102° F one hour later and gradually subsided (Fig 1). The remainder of the solution from the infusion bottle was cultured and found to be sterile. No further infusions were given this patient. The following day the patient developed a generalized papular eruption with pruritus. She complained of tender areas in the neck, and slightly enlarged, tender glands were palpable. The patient had been receiving a barbiturate *prn* during her hospitalization. Barbiturate medications were stopped. After several days the glands subsided and the rash disappeared with a dry, flaking desquamation. It was not established whether this cutaneous and glandular reaction was related to either the fat emulsion infusion or to barbiturate medication. The patient was discharged with her clinical condition unchanged.

**Patient 6** This patient was a 42-year-old housewife who entered the hospital with complaints of diarrhea and loss of weight. The diarrhea had grown progressively more severe during the past thirteen years. Except for occasional remissions—the last following therapy with folic acid—the disease had been refractory to all methods of therapy. At this time the patient was 50 per cent below her ideal weight. Her principal symptoms were weakness and tetanic cramps consequent to low serum levels of calcium. Nitrogen and fat balance studies indicated a great deficit in absorptive capacity although a definite reduction of pancreatic enzymes could not be confirmed. In the absence of a successful therapy for the fundamental disease, this patient represented an example of an urgent need for adequate parenteral nutrition, particularly calories. However, because of difficult veins, a considerable psychosomatic problem, and the fact that “pyrogenic difficulties” were shortly to plague the fat emulsions, it was not possible to give sufficient parenteral emulsion to make a real contribution to the patient's nutritional status. The subjective response to infusions of this patient was also difficult to interpret. The first infusion of Emulsion 41 to this patient was small, only 50 ml, but was given without any unfavorable reaction. After an interval



of one day a second infusion of the same emulsion in an amount of 150 ml was given with out reaction (see Table III, Patient 6 on 6/12 and 6/14). The following day a third infusion of the same emulsion in the amount of 150 ml was given and a mild pyrogenic reaction was obtained. On the next day the same batch of Emulsion 41 again was given to this patient in an amount of 300 ml and a pyrogenic reaction occurred with the temperature going to 102° F two hours after the infusion was finished.

TABLE III. CLINICAL STUDIES WITH FAT EMULSION 41

DATES 1948	AGE OF EMULSION (DAYS)	STUDIES	REACTION
4/30	0	Emulsion 41 Batch 9D 41—I prepared	0
		Sterility and pyrogen tests negative	0
5/10 to 5/23	11-24	Depressor test with cats negative	0
		Infusion into rats and dogs negative	0
5/24	25	Infusion Patient 1 (300 ml)	0
5/26	27	Infusion Patient 2 (300 ml)	0
5/27	28	Infusion Patient 2 (300 ml)	0
5/28	29	Infusion Patient 2 (300 ml)	0
5/29	30	Infusion Patient 2 (300 ml)	0
5/30	31	Infusion Patient 2 (300 ml)	0
5/31	32	Infusion Patient 2 (300 ml)	0
5/20	0	Emulsion 41 Batch 9D 42—I prepared	
		Sterility and pyrogen tests negative	0
5/21 to 5/23	1-8	Depressor test with cats negative	0
		Infusion into rats and dogs negative	0
6/1	12	Infusion Patient 2 (300 ml)	0
6/2	13	Infusion Patient 2 (300 ml)	0
6/12	23	Infusion Patient 3 (600 ml)	0
		Infusion Patient 4 (300 ml)	0
6/14	25	Infusion Patient 4 (300 ml)	0
		Infusion Patient 4 (300 ml)	0
		Infusion Patient 6 (300 ml)	0
		Infusion Patient 3 (300 ml)	0
6/15	26	Infusion Patient 4 (600 ml)	Pyrogenic
		Infusion Patient 6 (150 ml)	Pyrogenic
		Emulsion from infusion sets sterile	
6/16	27	Infusion Patient 3 (300 ml)	Pyrogenic
		Infusion Patient 4 (600 ml)	0
		Infusion Patient 6 (300 ml)	Pyrogenic
6/18	29	Infusion Patient 5 (300 ml)	Pyrogenic
		Emulsion from infusion sets sterile	+ rash
6/21	31	Sterility tests negative	0
		Pyrogen tests positive	Pyrogenic

Of all the patients, Patient 6 was the most sensitive to rate of infusion. Rates above 5 ml per minute led to nausea and on one occasion vomiting. At slower rates the subjective reaction was one of saturation and drowsiness.

Because of two consecutive pyrogenic reactions in Patient 6 no further infusions of fat emulsion were given to her at this time. However six weeks later additional infusions of Emulsion 41 were given again to Patient 6 but with no better results. In all Patient 6 was given 1500 ml of fat emulsion. Two months after the last infusion the patient expired and a post mortem examination was carried out. At death the patient was extremely cachectic. Examination revealed a granulomatous nonobstructing lesion of the bowel wall 45 cm below the pylorus. The etiology of this lesion is unexplained. Grossly and microscopically there was no evidence of the previous infusions of fat emulsion.

## DISCUSSION

When phosphatide fraction B(F-2) is used at a concentration of 0.5 to 1.0 per cent as the sole stabilizer of a 15 per cent fat emulsion, a good emulsion is obtained which will withstand autoclaving. This concentration of B(F-2) also produces minimal vasodepressor activity in the cat. It is felt, however, that such a low concentration of stabilizer may not be completely safe to protect the emulsion from breaking when it comes in contact with the electrolytes of the plasma. Hence a costabilizer of polyglycerol esters (Demal-14) has been used at a concentration of 1 per cent along with 0.5 per cent B(F-2) in a 15 per cent fat emulsion (Emulsions 40 and 41). A variety of studies with animals, using cats, rats, and dogs, indicated that except for a moderate temporary anemia Emulsions 40 and 41 appeared suitable for careful clinical trial.

Results on the first four patients were promising in that the emulsion was well tolerated with no changes in temperature, pulse, blood pressure, or respirations. Indeed, Patient 2 received 300 ml of Emulsion 41 (460 calories) per day for eight days in succession, and Patient 3 received an initial infusion of 600 ml of Emulsion 41 (920 calories) with no reaction and with definite clinical improvement.

The first batch of Emulsion 41 made up for clinical use was rather small, and when the preliminary trials with animals and sterility and pyrogen tests were completed, there remained seven 300 ml bottles of emulsion. These were completely used up in the first two patients, with no reaction (Batch SD-413, Table III). The second batch of Emulsion 41 (Batch SD-420) was made up in larger quantity and was used for the last two infusions of Patient 2 with no reaction. Likewise, this second batch of Emulsion 41 was given ten days later to Patients 3 and 6, and twelve days later to Patients 3, 4, and 6, all without reaction. However, on the following day (Table III, infusions on 6/15) pyrogenic reactions were obtained in two out of the three patients receiving this batch of fat emulsion. Since these were the first pyrogenic reactions observed, and since Patient 3 infused at the same time did not have a reaction, the significance of the pyrogenic reactions was questioned. Furthermore, emulsion from the infusion sets of all three patients was cultured and found to be sterile. Again on the following day two out of three patients had pyrogenic reactions, and two days later Patient 5 had a pyrogenic reaction (Table III, infusions on 6/16 and 6/18). Hence it appeared that something had developed in this batch of emulsion that caused a pyrogenic reaction. Three days following the last pyrogenic reaction in a patient, this batch of emulsion was found to be sterile but strongly pyrogenic in rabbits.

Thus it seems definite that the second batch of Emulsion 41 (SD-420) especially prepared for clinical use developed pyrogens of a nonbacterial nature twenty-six days after it was prepared, however, the first batch of Emulsion 41 (SD-413) remained pyrogen free for thirty-two days by which time the supply was exhausted (Table III). Additional studies have substantiated these results, namely, that while emulsions of the type of Emulsion 41, using B(F-2) at 0.5 per cent and Demal-14 at 1 per cent as stabilizers, can be used

satisfactorily in patients, the emulsions do undergo some type of change which gives rise to pyrogens. The exact cause or nature of this change is not understood at present. It does not seem to be due to contamination. It is accelerated by repeatedly autoclaving the emulsion.

In a discussion of the clinical use of fat emulsions it is desirable to caution against the possible instability of fat emulsions to electrolytes *in vitro*. Some of the emulsions prepared in this laboratory during the last few years have been found to be unstable when mixed with saline *in vitro*, however no instability was encountered with most of these emulsions when they were in contact with blood either *in vivo* or *in vitro*. Hence infusions of fat emulsions in man should not be given with an apparatus (flask, tubing or needle) through which saline has previously been given or vice versa unless the stability of the emulsion to saline has been established. That is why in the studies reported in this paper 5 per cent glucose in water (not saline) was the fluid used just prior to giving the fat emulsions.

#### SUMMARY

1 Fat emulsions for intravenous administration containing 15 per cent fat have been adequately stabilized with a combination of a soybean phosphatide preparation and a polyglycerol ester (Demul 14) at concentrations of 0.5 and 1 per cent respectively.

2 Such emulsions made with corn oil or coconut oil were well tolerated when given intravenously to rats, dogs and cats. Except for a moderate normocytic anemia which disappears shortly after the fat infusions are stopped, these fat emulsions gave rise to no apparent physiologic or pathologic difficulties, even after daily administration in generous amounts for periods of several weeks.

3 Such an emulsion containing 15 per cent coconut oil has been given to man in amounts up to 600 ml per day (940 calories) without unfavorable reactions. One patient received 300 ml of the emulsion daily for eight successive days without any unfavorable reaction. Post mortem examinations were later obtained on three of the six patients who received this emulsion and in no case was there any gross or microscopic evidence of pathologic changes related to the fat infusions.

4 Fat emulsions of the type used in these studies have been found to undergo some type of change over a period approximating four weeks that results in the formation of pyrogens and hence are not considered completely satisfactory for clinical use. The exact nature of this slow development of pyrogens is not known at present but it appears to be of nonbacterial origin.

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## LABORATORY METHODS

### THE DETERMINATION OF TRUE GLUCOSE IN BLOOD BY REDUCTION OF FERRICYANIDE

THE PRODUCTION OF A LINEAR REPRODUCIBLE COLOR WITHOUT THE USE OF A  
STABILIZING AGENT IN MACRO AND MICROTECHNIQUES SUITABLE FOR  
VISUAL AND PHOTOELECTRIC COLORIMETRY

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A SYSTEMATIC study of several blood glucose methods<sup>1</sup> employing colorimetric or photometric measurement of the reduction of alkaline ferricyanide indicated that further improvement and simplification of such methods was possible. A sensitive method yielding a color of sufficient stability and reproducibility for measurement in a photoelectric colorimeter without the need for frequent recourse to standard solutions and giving precise results over a wide range of true blood sugar values is described in this paper. The method has the additional advantage of being applicable directly to protein free filtrates of low dilution (1:10 macromethod) as well as high dilution (1:100 micromethod).

#### MICROMETHOD

*Reagents*—(Chemicals used are of analytical reagent quality unless otherwise noted.)

*Alkaline Potassium Ferricyanide* Weigh exactly 184 Gm. of anhydrous sodium carbonate and 98 Gm. of potassium ferricyanide and dissolve together in 1 liter of distilled water. Keep in dark bottle away from light.

*Ferric Iron* Dissolve exactly 20 Gm. of ferric ammonium sulfate ( $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ) in 945 ml. water and add 55 ml. of 85 per cent orthophosphoric acid.

*Acid Cadmium Sulfate* 130 Gm. cadmium sulfate ( $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ ) or 10.99 Gm. of anhydrous cadmium sulfate and 635 ml. of exactly 1N sulfuric acid are brought to a volume of 1 liter with distilled water.

*1.1N Sodium Hydroxide*

*Stock Glucose Standard* 1.000 Gm. made up to exactly 100 ml. with 0.25 per cent benzoic acid.

*Standardization*—0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 ml. of stock glucose standard are each diluted to 100 milliliters. 0.5 ml. of these standards is treated in the same manner as 0.5 ml. of the blood filtrate as outlined under Procedure for the micromethod. Readings may be plotted against concentration of glucose of the standards or a conversion factor (K) calculated:  $\text{milligrams glucose/100 ml.} = K(2 - \log \%T)$ . Readings  $D(-\log T)$  of blanks were found to vary from 0.01 to 0.03 with Coleman spectrophotometers and with scale readings of Klett Summerson instruments from 0 to 8. If blanks much higher than these are obtained the reagents are contaminated and should be discarded.

*Procedure*—To 8 ml. of acid cadmium sulfate solution add 1 ml. of whole blood mix thoroughly, let stand one minute then add 1 ml. of 1.1N sodium hydroxide and mix by shaking. Let stand fifteen minutes then filter through Whatman No. 40 filter paper or

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preferably centrifuge at 1000 r.p.m. for five to ten minutes. To a test tube graduated 20 ml. add 0.5 ml. of the filtrate, then add 5 ml. of the alkaline potassium ferricyanide solution. Mix by twirling and place in boiling water bath for ten minutes. Cool in a water bath at 40° C. Add 5 ml. of ferric iron solution and mix by twirling. Allow to stand five minutes in the 40° bath. Dilute to the 20 ml. mark with distilled water. Mix and read in the photoelectric colorimeter within twenty minutes. A green filter transmitting light between 500 and 570 mμ with maximum transmission at 540 mμ is used.

A more dilute filtrate may be prepared by adding 1 ml. of whole blood to 15 ml. of an acid cadmium sulfate solution prepared as follows: 5.77 Gm. cadmium sulfate ( $\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ ) or 4.7 Gm. anhydrous cadmium sulfate ( $\text{CdSO}_4$ ) and 28.2 ml. of exactly 1N sulfuric acid are brought to a volume of 1 liter with distilled water. One minute after the addition and mixing of the blood, 1 ml. of 11N sodium hydroxide is added and the procedure continued as directed.

1  $K = 1.5$  for Coleman spectrophotometers, Models 6 & 14 using No. 6304B cuvette.

#### MICROMETHOD

##### Reagents —

*0.1 per cent Stock Glucose Standard* Dissolve 100 mg. glucose and dilute to 100 ml. with 0.25 per cent benzoic acid.

*0.01 per cent Dilute Stock Glucose Standard* Dilute 10 cc. of stock standard to 100 ml. with 0.25 per cent benzoic acid.

*Alkaline Potassium Ferricyanide* Same as micromethod.

*Ferric Iron* Same as micromethod.

*Dilute Acid Cadmium Sulfate for 0.05 ml. Pipette* Dilute 40 ml. of reagent used in micromethod to 100 milliliters.

*Dilute Acid Cadmium Sulfate for 0.02 ml. Pipette* Dilute 20 ml. of reagent used in micromethod to 100 milliliters.

*0.055N Sodium Hydroxide for 0.05 ml. Pipette*

*0.022N Sodium Hydroxide for 0.02 ml. Pipette*

*Standardization*—Measure 2, 4, 6, 8, 10, 12, 15, and 20 ml. of dilute stock glucose standard with a burette and dilute to 100 ml. with 0.25 per cent benzoic acid. Add 0.5 or 1 ml. of these dilutions to colorimeter tubes and treat in the same manner as directed for 0.5 or 1 ml. of the blood filtrate in the microprocedures. One milliliter of 1 to 100 dilution or 0.01 per cent stock glucose standard is equivalent to 10 mg. of blood glucose if the micromethod for 0.02 ml. of blood is used, or 8 mg. if the micromethod for 0.05 ml. of blood is used. Readings may be plotted against concentration of glucose of the standards or a conversion factor ( $K$ ) calculated: milligrams glucose/100 ml. =  $K(2 \log\%T)$ .

*Procedure for 0.02 ml. Pipettes*—Add 0.02 ml. of whole blood to 1 ml. of dilute acid cadmium sulfate solution for 0.02 ml. pipettes in a small Klett tube and mix by washing out pipette. Let stand for one minute. Add 1 ml. of 0.022N sodium hydroxide, stopper and mix.

1  $K = 503$  in the micromethod for 0.02 ml. of blood for Coleman spectrophotometers, Models 6 & 14, using No. 6304B cuvettes.

Let stand fifteen minutes, then centrifuge. Add 1 ml. of the clear supernatant filtrate to 15 ml. of alkaline potassium ferricyanide in a colorimeter tube graduated at 6 milliliters. Digest ten minutes in a boiling water bath. Cool two minutes in a 40° C. water bath. Add 1.5 ml. of the ferric iron solution, mix and allow to stand five minutes at 40°. Dilute to the 6 ml. mark and read in the colorimeter within twenty minutes. Make reagent blank by treating 1 ml. of distilled water in the same manner as 1 ml. of filtrate and set at 100 per cent transmission with light at 740 mμ wave length.

*Procedure for 0.05 ml. Pipettes*—Add 0.05 ml. of whole blood to 1 ml. of the dilute acid cadmium sulfate solution for 0.05 ml. pipettes in a small centrifuge tube and mix by washing out pipette. Let stand for one minute. Add 1 ml. of 0.055N sodium hydroxide, mix and stopper. Let stand fifteen minutes then centrifuge. Add 0.5 ml. of the supernatant filtrate to 1.25 ml. of alkaline potassium ferricyanide in a 5 ml. graduated Klett colorimeter tube. Digest ten minutes in a boiling water bath. Cool two minutes in a 40° C. water bath.

Add 1.25 ml of the ferric iron solution mix and allow to stand five minutes at 40° C. Dilute to the 5 ml mark and read in colorimeter within twenty minutes as directed in preceding meromethod.

#### EXPERIMENTAL

1 *Determination of Optimum Concentration of Reagents and Physical Conditions for the Production of a Stable Reproducible Linear Color*—Optimum concentrations of reagents were determined by studying the effects of variation in their composition and proportion on color development in several ferric cyanide reduction sugar methods. Tungstic acid filtrates of blood were employed. Whatman No. 40 filter paper free of reducing substances was used. Substitute reagents also were studied with the hope of discovering more desirable ones. It was found that a marked reduction in the concentration of sodium carbonate and phosphoric acid is compared with previous methods greatly increased the speed of the development and improved the stability of color. Lithium carbonate was found to exhibit the same characteristics as sodium carbonate when used in equivalent concentrations. Phosphoric acid in excess of the optimal concentration diminished the stability of color in the older ferric cyanide methods. A potentiometric study by Wood<sup>8</sup> of the oxidation of glucose by ferric cyanide has shown that increase of acidity retards the reaction. The concentration of other reagents was also reduced from that recommended by Folm and Malmros.<sup>1</sup> Cyanide carbonate reagent recommended by Horvath and Knehl, has been shown by Suter<sup>7</sup> and others to increase progressively for forty five minutes the ferricyanide formation when heated. Small variations in the amount of sodium cyanide added also cause variations in the development of color. High values for blanks are common when cyanide is used and the range of the method is reduced. For these reasons sodium cyanide was omitted from the alkaline ferricyanide reagent. In agreement with Klendshoj and Hubbard<sup>4</sup> and Horvath and Knehl ferric ammonium sulfate was found to develop a more stable color over a greater range of concentration than other ferric compounds tested. These included ferric sulfate phosphate trihydrate and citrate. A 0.2 per cent ferric ammonium sulfate ( $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ ) solution was found to give a linear relationship between color and glucose concentration up to 400 mg per 100 ml of glucose at any time after color was developed at room temperatures. The color gradually increased by about 5 per cent in twenty five minutes, then remained stable for more than one hour. The disadvantage of increasing color intensity after dilution to volume was overcome by warming five minutes at 40° after addition of the ferric ammonium sulfate to the reaction mixture. When so treated the solution could be read in the colorimeter immediately after diluting to volume and mixing. Only occasional samples showed an increase of 1 to 2 mg per 100 ml after standing twenty minutes. Ferric ammonium sulfate concentrations exceeding the 0.2 per cent recommended cause slow fading of color while less caused intensity of color to increase on standing.

All previous methods depending upon the formation of Prussian blue have used a stabilizing agent. When higher concentrations of reagents were used sodium dimethyl sulfonate (Diponol) was found to stabilize the colloidal pigment

which otherwise precipitated when higher levels of glucose were examined. However, different samples of Duponol "p e" or "e" gave somewhat different blanks and caused a variation in time for full color development. When the concentration of reagents was reduced to that recommended in this paper it was found that the addition of Duponol to the ferric ion reagent did not increase the stability of the color developed, but prolonged the time required for its development and also increased the color of the blank. For these reasons the use of a stabilizing agent was abandoned.

Tungstic acid filtrates examined by the modified method gave results about 10 per cent higher than the Folin and Malmros<sup>1</sup> (2 to 33 mg per 100 ml) and Benedict<sup>9</sup> (0 to 40 mg per 100 ml) methods. A series of fifteen samples were used to compare the variability of the proposed ferricyanide method and the Folin and Malmros<sup>1</sup> method by three repeated determinations on preserved tungstic acid filtrates. Single determinations by the new method deviated from the mean by an average of 3.3 mg per 100 ml ranging from 0 to 7 mg on samples with blood sugars of 55 to 150 mg per 100 milliliters. On the same filtrates, the Folin and Malmros method showed an average deviation from the mean of 12.6 mg per 100 ml ranging from 4 to 21. The color developed by the Folin and Malmros<sup>1</sup> and the Benedict<sup>9</sup> methods was less stable and therefore less satisfactory for photoelectric determination than that developed by the methods herein described.

No special tubes are required for the new ferricyanide method, and as a minimal amount of sodium carbonate is used, gas bubble formation is avoided. No differences have been found after the reagents have been aged for about two months in contrast to the Folin and Malmros<sup>1</sup> and Benedict<sup>9</sup> reagents which undergo changes in sensitivity on standing.

*2 Determination of Optimum Concentration of Reagents and Physical Conditions for the Preparation of Protein-Free Filtrates for the Determination of "True" Glucose*—Although consistent results were obtained with tungstic acid filtrates, it was felt that the method would be more useful if true sugar values could be obtained. Protein-free centrifugates and filtrates prepared by various methods were studied with the modified ferricyanide method. Protein-free filtrates prepared from serum by copper-tungstate precipitation gave results comparable to tungstate filtrates. The copper-tungstate reagent could not be used on whole blood because stability of color was not obtained. Zinc or barium hydroxide could not be used for the same reason. However, acid cadmium sulfate and sodium hydroxide could be adapted to the ferricyanide method by modification of the original procedure of Fujita and Iwatake.<sup>6</sup>

A comparison of tungstic acid and cadmium hydroxide filtrates was made, and the nonfermentable substances (Table I) of the filtrates were determined. 0.5 ml of a 50 per cent suspension of yeast cells, washed free of reducing substances, was added to 5 ml of the cadmium filtrates in a centrifuge tube and incubated at 38° for thirty minutes and then centrifuged. The unfermentable reducing substance was found by this method to average 3 mg per 100 ml in cadmium hydroxide-treated specimens as compared with 25 mg per 100 ml expressed as glucose in tungstate-treated specimens.



TABLE I COMPARISON OF TOTAL REDUCING SUBSTANCES IN CADMIUM HYDROXIDE AND TUNOSTIC ACID FILTRATES AND NONFERMENTABLE REDUCING SUBSTANCES IN CADMIUM HYDROXIDE FILTRATES AS DETERMINED BY THE NEW FERRICYANIDE METHOD

NO	TUNOSTIC ACID FILTRATES	CADMIUM HYDROXIDE FILTRATES	
	TOTAL REDUCING SUBSTANCES (MG %)	TOTAL REDUCING SUBSTANCES (MG %)	NONFERMENTABLE REDUCING SUBSTANCES (MG %)
1	78	51	3
2	82	50	2
3	115	83	4
4	122	93	3
5	86	60	3
6	90	52	3

The effects of cadmium were evaluated by adding graduated amounts of glucose to a series of different cadmium hydroxide prepared protein free filtrates. Recoveries indicated that the amount of cadmium sulfate recommended did not interfere even if added in 5 to 10 per cent excess unless the protein content of the sample is very low (less than 4 per cent serum proteins or 2 million red blood cells), when excess cadmium may cause turbidity to occur during color development. In conditions of this kind turbidity may be avoided by reducing the protein precipitants twenty five per cent. Standard solutions of glucose added to whole blood (with correction for glucose present in the blood) gave readings identical with those of glucose standards in water. Further tests of a possible interfering action of cadmium were made on filtrates obtained by combining acid cadmium sulfate and sodium hydroxide in the proportions used for protein precipitation the addition of blood being omitted. If 0.5 ml of 1 to 6 or 1 to 8 dilutions of such filtrates was added to glucose standards no effect on final color development was observed. Cadmium hydroxide filtrates of blood may be kept several days at 5° C without measurable loss of sugar.

TABLE II RECOVERIES OF GLUCOSE ADDED TO 1 ML. OF OXALATED WHOLE BLOOD\* WITH ACID CADMIUM SULFATE REAGENT†

NO	GLUCOSE IN 100 ML. OF BLOOD (MG)	GLUCOSE ADDED (MG)	GLUCOSE RECOVERED (MG)	PER CENT RECOVERED
MACROMETHOD				
1	43	0.495	0.50	101.0
2	50	0.980	1.00	102.0
3	38	0.980	0.97	99.0
4	67	1.455	1.53	105.1
5	49	1.920	1.91	99.5
6	59	1.920	1.83	95.3
7	54	2.380	2.42	101.6
8	67	0.495	0.48	96.9
9	37	2.580	2.40	100.8
MICROMETHOD				
RECOVERIES OF GLUCOSE ADDED TO 0.05 ML. WHOLE BLOOD*				
1	37	0.0180	0.0175	97.2
2	67	0.0477	0.0495	103.8
3	53	0.0477	0.0490	102.7
4	51	0.0930	0.0880	94.6
5	40	0.1364	0.1300	95.3

\*Blood specimens from hospital patients. No attempt was made to preserve the blood sugar prior to analysis. These specimens had stood several hours after blood was collected.  
†Glucose was dissolved in the acid cadmium sulfate solution.

3 *Determination of the Accuracy of the New Method, and the Normal "True" Glucose Values Obtained by It*—Known amounts of glucose were added to specimens of oxalated whole blood and the recoveries determined (Table II). The data from these recoveries indicated that the glucose found in alkaline cadmium sulfate filtrates does not vary by more than 3 mg per cent from the true blood sugar value. Recoveries within 94.6 to 105.1 per cent were obtained.

TABLE III COMPARISON OF THE NEW FERRICYANIDE AND THE FOLIN MALMROS BLOOD SUGAR METHODS USING CADMIUM HYDROXIDE FILTRATES FROM FRESH OXALATED WHOLE BLOOD\* AND PLASMA\* FROM HOSPITAL PATIENTS

NO	PLASMA		BLOOD		(CELLS†)		PACKED CELL VOLUME (%)
	FERRI CYANIDE (MG %)	FOLIN MALMROS (MG %)	FERRI CYANIDE (MG %)	FOLIN MALMROS (MG %)	FERRI CYANIDE (MG %)	FOLIN MALMROS (MG %)	
1	80	72	70	70	64	63	40.3
2	96	87	71	82	57	79	44.3
3	85	75	62	68	47	64	36.4
4	61	68	61	65	61	63	39.6
5	58	54	43	47	34	43	38.9
6	127	116	103	98	89	87	39.0
7	50	59	51	54	51	50	26.2
8	69	63	72	61	73	59	34.7
9	125	118	105	104	94	96	45.2
10	77	75	66	68	59	64	40.4
11	115	100	117	112	117	114	14.0
12	63	64	58	54	56	49	52.0
13	75	73	57	68	47	65	49.5
Average	83	79	72	73	65	69	38.3

\*Fasting

†Calculated from cell volume

Fasting venous blood glucose was determined on the plasma and whole blood of thirteen hospital patients by the new ferricyanide method and the Folin and Malmros method using cadmium hydroxide filtrates (Table III). Glucose from the cells was calculated by means of hematocrit determinations. The glucose values by the Folin and Malmros method averaged 4 mg per cent lower in plasma and 4 mg per cent higher in cells, differences that are probably not significant. It is believed, as previously stated, that the large individual differences result from the lower precision of the Folin and Malmros method which has not shown good agreement between repeated determinations. Values for nonfermentable reducing substances obtained by the two methods on cadmium filtrates were the same. It was shown by experiment that the Folin and Malmros method was unaffected by amounts of cadmium equivalent to that in the protein-free filtrates.

The new ferricyanide method with cadmium hydroxide filtrates and the Folin-Wu method<sup>10</sup> with acid tungstate filtrates were compared, using venous blood from twenty-three normal fasting male subjects (Table IV). Glucose ranged from 73 to 85 mg per 100 ml of venous blood as determined by the new ferricyanide method, which is slightly lower, but agrees favorably, with the range of 77 to 90 obtained by Somogyi<sup>11</sup> on the venous blood of ninety-three fasting normal individuals. A range of 85 to 110 mg of glucose per 100 ml of venous blood was obtained by the Folin Wu method. The latter method was used for comparative purposes as it is probably the most widely used of all sugar methods in clinical laboratories.

TABLE IV COMPARISON OF THE NEW FERRICYANIDE (CADMIUM HYDROXIDE FILTRATES) AND THE FOLIN WU (ACID TUNGSTATE FILTRATES) BLOOD SUGAR METHODS USING FILTRATES FROM FLESH OXYLATED WHOLE BLOOD OF NORMAL FASTING (12 TO 14 HR.) MEN 19 TO 39 YEARS OLD

SUBJECT	FERRICYANIDE (MG %)	FOLIN WU (MG %)
BUR	81	100
RAS	81	98
HIG	75	98
SHW	74	84
JAR	84	100
DIM	81	94
SHE	74	100
SEN	84	100
MCC	71	85
BRO	84	96
KIN	81	93
LOG	82	99
CAL	82	93
MAC	85	100
LEH	82	96
SHA	75	95
LAC	81	110
BUD	85	87
STA	81	90
STO	81	101
SNI	83	86
CAS	82	110
ALA	85	108
Average	81	97

## SUMMARY

A method for the photocolormetric determination of 'true sugar' in whole blood is described. The conditions necessary for obtaining a stable, linear reproducible color for the quantitative determination of glucose by its reduction of ferricyanide in alkaline solution are outlined.

A normal fasting glucose concentration of 73 to 85 mg per 100 ml of venous blood was obtained.

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# SIMPLE TEST FOR THE APPROXIMATE ESTIMATION OF BLOOD CREATININE AND GLUCOSE IN ONE PROCEDURE

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THE use of picric acid as a protein precipitating agent in the determination of creatinine in the blood was recommended previously by one of the authors (E E M) in collaboration with others<sup>1,3</sup> During a recent re-evaluation of the bedside modification of that method<sup>4</sup> it was found that the brief heating of the blood-picric acid mixture as prescribed at times resulted in a filtrate which yielded a much more intense color reaction than warranted by the true creatinine content, as determined with the routine precision method. This phenomenon was attributed to the glucose present in the blood, in accordance with reports long since published<sup>5,8</sup> Hence, it was not only obvious that the original technique of quick creatinine estimation<sup>4</sup> needed revision, but it also appeared that the same method might lend itself to a simultaneous determination of the blood sugar. This notion was verified by subsequent experiments, the results of which are outlined below.

## REAGENTS

- (a) Saturated picric acid
- (b) 10 per cent sodium hydroxide

## APPARATUS

- (A) Standardized test tubes approximately 5 inches long and  $\frac{1}{2}$  inch in diameter, graduated at 1, 4, 5, and 10 milliliters
- (B) Small funnel fitted with folded filter paper (Whatman No 1)
- (C) Medicine droppers
- (D) Boiling water bath (cup or beaker)
- (E) Potassium bichromate standards ranging from 0.15 to 3 per cent solutions (Table I) contained in test tubes identical with those described under A, but not graduated, and sealed with paraffin. They are prepared from a 10 per cent solution, acidified with 1 drop of concentrated sulfuric acid per 100 ml, by progressive dilution with distilled water, and are stable for years if kept in a cool and dark place.
- (F) Three slot comparator with ground glass background to be viewed against daylight or an artificial daylight bulb.

## PROCEDURE

*Creatinine Estimation*—Fill a graduated test tube (A) with reagent (a) to the 4 ml mark. Add venous blood with a syringe or a medicine dropper up to the 5 ml mark, using either a freshly drawn or a preserved specimen rendered noncoagulable. Shake thoroughly and let stand for five minutes or until the reddish brown color of the mixture changes to greenish brown. Filter into a similar test tube until the clear yellow filtrate reaches the 1 ml mark. Adjustments of volume can be made readily with a medicine dropper. Add 1 drop of reagent (b), shake, and allow fifteen minutes for development of yellow orange color.

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Read in comparator against bichromate standards for creatinine estimation (Table I). In the absence of such standards, comparison with a specimen known to contain a normal concentration of creatinine, or with a simple mixture of 1 ml of reagent (a) with 1 drop of reagent (b), may be utilized. An intensity of color in excess of that of either improvised standard denotes an abnormal creatinine level. An increase of 3 mg per cent or more can be detected readily without the aid of a comparator.

TABLE I CORRELATION OF GLUCOSE AND CREATININE LEVELS WITH CONCENTRATION OF POTASSIUM BICHROMATE STANDARDS

BLOOD CREATININE (MG PER 100 ML)	POTASSIUM BICHROMATE (MG PER 100 ML)	BLOOD GLUCOSE (AT AVERAGE CREATININE LEVEL OF 1 MG %) (MG PER 100 ML)
0.8	1.0	50
1.0	200	67
1.6	250	78
2.0	300	90
2.3	350	95
2.6	400	100
3.2	500	112
3.7	600	120
4.2	700	130
4.6	800	138
5.0	900	145
5.4	1000	150
5.7	1,100	155
6.0	1200	160
6.4	1300	165
6.8	1400	170
7.1	1500	175
7.5	1,700	180
8.0	1900	185
8.4	2000	190
9.1	2200	195
9.8	2400	200
10.2	2,500	205
14.0	3000	215

*Glucose Estimation*—Having served for the visual creatinine determination the same specimen is immersed in a boiling water bath for about three minutes. A change in color to a more or less intense reddish brown occurs depending upon the concentration of glucose present. Periods of two to ten minutes used for boiling the specimen produce the same results both visual and spectrophotometric. The glucose level can be estimated roughly immediately after removal of the tube from the water bath by one who has gained some experience with the test.

For better accuracy add distilled water to the 5 ml mark and after mixing, compare the resulting color with bichromate standards as listed in Table I. With color reactions more intense than that of the 2400 mg per cent bichromate solution (above 200 mg per cent of glucose), dilute the specimen with another 5 ml of water by running up to the 10 ml mark. Without this additional dilution of samples with higher glucose contents the deepness of their color renders visual comparison unreliable and does not permit accurate spectrophotometric correlation with potassium bichromate standards (see below). Doubling of the value obtained for the diluted specimen from Table I yields the true blood glucose level. Further dilution (replacing 5 ml of the 10 ml specimen with water) and quadrupling of the result is necessary for blood samples with a sugar content greater than 400 mg per cent.

With these higher dilutions, the influence of an elevated creatinine on the glucose reaction is negligible. They were not employed with lower glucose concentrations because

If the capacity of the test tubes (A) used is less than 10 ml, transfer 1 ml. of the specimen to another tube (A) and make up with distilled water to the 2 ml mark prepared for this purpose. Fourfold dilution if necessary is implemented by adding water up to the 4 ml mark.

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# A QUANTITATIVE SPINAL FLUID GLUCOSE MICROMETHOD FOR THE PEDIATRIC WARD LABORATORY

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IT HAS been well established that a lowered glucose concentration in spinal fluid is an important clue in the early diagnosis of meningitis,<sup>1,4</sup> particularly when other spinal fluid findings are lacking. Since this diagnosis frequently must be established at night when laboratory facilities are limited or unavailable, reliance is often put on the qualitative reaction of Benedict's solution.<sup>5</sup> This reaction is difficult to evaluate quantitatively and requires 0.75 ml to 1.0 ml of spinal fluid, an amount unavailable from some infants.

To provide a rapid quantitative estimation of spinal fluid glucose the following adaptation of Lewis and Benedict's picric acid method for glucose<sup>6,7</sup> has been employed. A single 0.25 ml sample of spinal fluid suffices for the determination. No colorimeter is required. Results are expressed as milligrams per cent glucose and usually do not vary from results obtained with the Folin ferrieyanide method<sup>8</sup> by more than 5 mg per cent.

## METHOD

**Reagents**—Sodium picrate 10 per cent aqueous solution. This is prepared free of the red colored picramic acid as described by Folin.<sup>10</sup>

Sodium carbonate 10 per cent aqueous solution. This and the sodium picrate solution are stored in gravity flow stock bottles with dropper tips on the tubing.

Standard glucose solution 200 mg. per cent. Ten additional dilutions from 10 to 100 mg. per cent are made from this stock solution.

**Apparatus**—Permanent color standards. These are easily made from 10 by 75 mm soft glass test tubes. Tubes for permanent standards and for the reaction should be of the same inside diameter. This may be measured with a caliper or by delivering water into several tubes from a 10 ml pipette. Those with the same water level are selected. The upper portion of the test tube is drawn out to a tip which is broken off. Permanent colored solutions are made by adding equal quantities of glucose standard and dilutions 10 per cent sodium carbonate solution, and 10 per cent sodium picrate solution to a test tube. This is placed in boiling water for eight minutes. The eleven colored solutions so prepared are introduced by dropper into the drawn out tips of the 10 by 75 mm test tubes which are sealed off in a flame. This makes eleven permanent standards between 10 and 200 mg. per cent glucose which may be kept in a wooden comparator block.

Reaction tubes. 10 by 75 mm Pyrex test tubes are used.

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†Transfer 500 Gm. moist picric acid to a Florence flask of 1.5 liters capacity. Add 50 ml. of acetone. Shake with a little warming under hot tap water until all the crystals are dissolved. Dissolve 250 Gm. of anhydrous sodium carbonate and 100 Gm. of sodium chloride in 7.5 liters of warm water in a 4 liter beaker. While stirring with a large agateware spoon add the acetone solution gradually to the alkaline salt solution. When the reaction is finished let stand in cold water for about half an hour and then gradually pour the now nearly solid mass on a hardened filter on a large Buchner funnel (4 cm. diameter). Wash the picrate with about 5 liters of 5 to 10 per cent sodium chloride solution. Dry at room temperature or over a radiator. Make to 10 per cent aqueous solution.



Fig 1—An easily made comparator block for the permanent color standards. Attached cross wise is a reaction tube with rings scored at 0.25 ml, 0.50 ml, and 0.75 milliliter.

*Procedure*—From a graduated 1 ml pipette add 0.25 ml spinal fluid to a reaction tube. Then add 0.25 ml 10 per cent sodium picrate solution and an equal quantity of 10 per cent sodium carbonate solution. An alternative procedure, which avoids the use of pipettes, is to calibrate the reaction tubes by scoring them with a diamond pencil at 0.25 ml, 0.50 ml, and 0.75 ml. The spinal fluid and solutions may then be added from clean medicine droppers. Shake. Place in boiling water eight minutes. Put in comparator block and compare with permanent standards. Express results as milligrams per cent glucose.

#### RESULTS

Table I presents a comparison of spinal fluids analyzed for glucose by Folin's ferricyanide method and by the picrate method. The data show that variation in spinal fluid total protein does not influence the similarity of results with the two methods.

Creatinine reacts with sodium picrate to produce a color similar to that produced by glucose. Table II shows a comparison of spinal fluids analyzed

TABLE I. COMPARISON OF ANALYSES OF SPINAL FLUID FOR GLUCOSE BY THE FOLIN FERRICYANIDE METHOD AND BY THE PICRATE METHOD

TOTAL PROTEIN (mg %)	GLUCOSE (mg %)	
	FERRICYANIDE METHOD	PICRATE METHOD
16.8	64.5	65
41	62.1	60
28	55.0	55
40	94.7	100
31	58.8	55
45	55.0	55
96	68.9	70
23	47.0	50
58	52.6	55
52	60.0	70
80	54.6	60
56	66.0	70
80	42.0	35

Average difference of picrate method values from ferricyanide method values =  $\pm 3.47$  mg per cent glucose



TABLE II. COMPARISON OF ANALYSES OF SPINAL FLUID FOR GLUCOSE BY THE PICRATE METHOD WITH AND WITHOUT CREATININE IN A CONCENTRATION OF 5 MG PER CENT

TOTAL PROTEIN (MG %)	FOLIN CIANIDE METHOD GLUCOSE (MG %)	PICRATE METHOD GLUCOSE (MG %)	
		NO CREATININE	CREATININE ADDED
38	74.0	80	80
30	66.6	70	70
45	66.0	70	70
128	66.2	70	70
84	27.7	—	40

for glucose by the picrate method with 5 m. per cent creatinine added to one group. This concentration was found by Myers and Fine only in severe nephritis.<sup>11</sup> Comparison of the two groups shows similar results for glucose except in the spinal fluid containing least glucose where the picrate method gave a higher reading. Hence in the rare patient with both meningitis and severe nephritis this error might occur.

In the absence of meningitis spinal fluid sugar reflects hyper and hypoglycemia.<sup>1-13</sup> The 200 mg per cent standard was included to detect hyperglycemia if it exists.

#### SUMMARY

A modification of the Lewis and Benedict picric acid method for glucose is described for use with spinal fluid requiring only 0.25 ml of spinal fluid. No colorimeter is required.

Results agree closely with the Folin ferricyanide method for glucose (average difference  $\pm 3.47$  m. per cent). The condition in which spinal fluid creatinine may cause error is discussed.

The authors are indebted to Dr. Allan M. Butler for helpful suggestions.

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## ACID PHOSPHATASE TEST FOR IDENTIFICATION OF SEMINAL STAINS

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A TEST for the identification of seminal stains as a means of determining the accuracy of charges of rape must have a high degree of reliability with almost no margin of error

The isolation of spermatozoa properly fixed and stained is by far the most satisfactory test for the demonstration of male ejaculate. However, chemical identification for the presence of seminal fluid adds further proof. In some instances chemical identification may be of more importance since it is not always possible to demonstrate the presence of intact spermatozoa with both head and tail, or even to identify microscopically the separate parts with certainty. In old specimens especially, some of the tails may be missing or abnormally twisted and the disjointed sperm heads are extremely difficult to identify and may be confused with spores, pus cells, or artifacts. In such situations and in aspermia or azoospermia\* in which the ejaculate lacks spermatozoa, other reliable microchemical tests are desirable.

The first microchemical test for seminal stains, described by Florence in 1896, has been used generally and accepted by scientific laboratories and law enforcement agencies. The procedure is simple, using a modified Wagner's reagent (16 Gm potassium iodide, 25 Gm iodine, dissolved in 30 ml water). At the line of confluence between the drop of extract and reagent, dark rhombic crystals of choline iodide may be microscopically observed. Results with the Florence test are not conclusive and occasionally may be erroneous.<sup>6b</sup>

If soap is used in the extraction, the test may be negative, moisture and putrefaction are sources of error. Albumin, glycerophosphoric acid, choline, trimethylamine, and extracts of mushrooms, corn, blossoms of plants, onions, and many other materials may interfere or give false positive results. Many attempts have been made to modify the Florence test to increase its specificity but none of the modifications are reliable. The subject has been adequately reviewed by Pollak.<sup>6a</sup>

It is first demonstrated that dried seminal stains give a characteristic fluorescence under ultraviolet light. However, this same brilliant characteristic fluorescence also may be obtained by many other substances and is therefore nonspecific for semen. It is of some value in locating suspected stains over large areas of garments and then marking these for later extraction.

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\*Aspermia is the congenital or acquired obstruction of the ducts preventing passage of sperm from the sex glands. azoospermia is the condition of immature cells of spermatogenesis which may be temporary or permanent due to frequent intercourse alcoholism or internal disease.<sup>6a 6b</sup>

or tactile perception or from ultraviolet light observation that the stain is widely and thinly dispersed or diluted the size of the area to be extracted should be increased. Body fluids and the common stains will not interfere since it was found that even in concentrated form these do not have an increased acid phosphatase activity. (See Table I)

Since the usual common contaminants that may simulate the appearance of seminal stains all have a relatively low acid phosphatase activity (Table I) and stains produced by semen have such a high acid phosphatase activity there is still a wide margin for error allowed if the minimum value for interpretation of a positive test is set at 25 units per milliliter of solution.

In the past occasion has presented itself where the precipitin test of Latham may have been performed to differentiate between stains of human or animal origin. It is believed that if the possibility of the stain being of monkey origin can be ruled out the positive acid phosphatase test would suffice to indicate a prostatic secretion of human origin.

#### SUMMARY

In the legal proof of rape scientific evidence must be specific and should allow no margin of error.

The microchemical tests including the Florence test heretofore used in the identification of seminal stains have been nonspecific.

A microchemical test suggested by Hansen and Rusfeldt is described based on the high acid phosphatase level of seminal fluid of human origin. This promises to be both a specific and sensitive procedure for the positive identification of seminal stains as an adjunct to the demonstration of whole spermatozoa and of particular value in cases of aspermia or azoospermia.

#### CONCLUSIONS

1 The acid phosphatase method of recognition of seminal stains is particularly good in that differences between positive and negative results are easily interpreted.

2 The reaction is very sensitive and appears to be specific for seminal fluid of man or monkey only. Alkaloids, common food stains and body fluid stains do not interfere with the test.

3 The test can be performed simultaneously with the demonstration of fixed and stained spermatozoa without loss of material tested.

4 Seminal stains give positive tests even after six months.

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specimen with 3 ml of water, repeat the extraction and tease the sample with a small medicine dropper, using the same 3 ml of original water. Allow sufficient time for a complete extraction, then perform an acid phosphatase determination on 1 ml of the filtered extract. It is even better to centrifuge the extract, examine the residue, and stain for spermatozoa in an accepted manner (Giemsa or Gram stain is satisfactory), in addition to doing an acid phosphatase test on the supernatant fluid. These tests may be performed simultaneously with the same original extract.

The procedure used for these experiments is a modification of the King-Armstrong method described by the Paul Lewis Laboratories, however, any accepted procedure for the determination of acid phosphatase is satisfactory, but particular attention should be given to the comparative results and technique employed (incubation time, reagents, etc.). In positive cases where the samples examined were sufficient, in no instance were the high values doubtful as to interpretation, regardless of results or technique used. A control should be simultaneously run to rule out possible interference. Positive results are indicated by the marked, increased acid phosphatase activity in comparison with the low acid phosphatase activity of body and food contaminants as shown in Tables I and II.

TABLE II ACID PHOSPHATASE VALUES FOR SEMINAL FLUIDS

	KING-ARMSTRONG UNITS
Seminal fluids	
Fresh	2,000-2,800 per ml
Decomposed	800-1,700 per ml
Seminal stains	
Dissolved in 5% KCN	< 50 per sq cm *
Dissolved in 3% NaF	< 50 per sq cm
Heated at 100 C for 3 min	< 50 per sq cm
1 day old	> 50 per sq cm
1 week old	> 50 per sq cm
1 month old	> 50 per sq cm
6 months old	> 50 per sq cm

\*Of cloth stained with seminal fluid and dried

The color intensity obtained with seminal stains is too great to read directly. Consequently, dilution of the final color with distilled water is necessary in order to approximate the color intensities of standards calibrated for colorimetric readings. The dilution factor must be taken into consideration when making the final calculations.

It is not always practical or possible to extract a definite unit area as was previously suggested since absorbent properties cannot always be taken into consideration of the different cloths and stains on materials such as grass, soil, wood and rocks. Also, inasmuch as it is reasonable to assume that the area concentration of a stain will be unevenly distributed with its highest concentration toward the center of the dried stain, judgment should be used in determining the size of the sample. An area of approximately 1 sq cm usually will suffice.

#### INTERPRETATION

An acid phosphatase activity of 25 King-Armstrong units per milliliter of extract from an area of approximately 1 sq cm should be considered positive for seminal stains. This value was arbitrarily established by observation and comparison with stains of fruit, vegetables, body fluids, and with stains produced by semen. With seminal stains, conditions may be quite varied, depending upon size, unit concentration, thickness, complete extraction, dilution factors produced by rain, washing, or contaminants. These variables are difficult to control or estimate accurately. Therefore, good judgment is necessary in determining the size of the sample to be taken. If it is obvious from visual

or tactile perception or from ultraviolet light observation that the stain is widely and thinly dispersed or diluted the size of the area to be extracted should be increased. Body fluids and the common stains will not interfere since it was found that even in concentrated form these do not have an increased acid phosphatase activity. (See Table I.)

Since the usual common contaminants that may simulate the appearance of seminal stains all have a relatively low acid phosphatase activity (Table I) and stains produced by semen have such a high acid phosphatase activity there is still a wide margin for error allowed if the minimum value for interpretation of a positive test is set at 25 units per milliliter of solution.

In the past occasion has presented itself where the precipitin test of Gammum may have been performed to differentiate between stains of human or animal origin. It is believed that if the possibility of the stain being of monkey origin can be ruled out the positive acid phosphatase test would suffice to indicate a prostatic secretion of human origin.

#### SUMMARY

In the legal proof of rape scientific evidence must be specific and should allow no margin of error.

The microchemical tests including the Florence test heretofore used in the identification of seminal stains have been nonspecific.

A microchemical test suggested by Hansen and Rinsfeldt is described based on the high acid phosphatase level of seminal fluid of human origin. This promises to be both a specific and sensitive procedure for the positive identification of seminal stains as an adjunct to the demonstration of whole spermatozoa and of particular value in cases of aspermia or azoospermia.

#### CONCLUSIONS

1 The acid phosphatase method of recognition of seminal stains is particularly good in that differences between positive and negative results are easily interpreted.

2 The reaction is very sensitive and appears to be specific for seminal fluid of man or monkey only. Alkaloids, common food stains and body fluid stains do not interfere with the test.

3 The test can be performed simultaneously with the demonstration of fixed and stained spermatozoa without loss of material tested.

4 Seminal stains give positive tests even after six months.

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#### ERRATUM

In the article by Allen and associates, "A Protamino Titration as an Indication of a Clotting Defect in Certain Hemorrhagic States," in the April issue, the first line, p 473, should read Dogs exposed to 450 r as total body x ray develop a clotting defect similar to that produced by the intravenous injection of heparin <sup>1</sup>

## CULTIVATION OF MYCOBACTERIUM TUBERCULOSIS

### A COMPARISON OF HYDROCHLORIC ACID AND TRISODIUM PHOSPHATE IN THE PREPARATION OF SPUTUM AND OF THE USE OF FLUID DUBOS DAVIS MEDIUM AND PETRAGNANI MEDIUM

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IN 1946 Cooper and Stoner<sup>1</sup> described the use of trisodium phosphate in the preparation of sputum for the diagnostic culture of mammalian types of *Mycobacterium tuberculosis*. These workers reported that this organism was not destroyed by contact with a 10 per cent solution (23 per cent  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ ) of trisodium phosphate for a week at room temperature and that contaminating organisms in sputum were destroyed without hazard to the tubercle bacillus by contact for twenty four hours at 37° C. Van Vranken reported a comparison of the use of oxalic acid and trisodium phosphate in the treatment of sputum specimens for culture. She found that the use of trisodium phosphate yielded more positive cultures than the use of oxalic acid both when acid fast organisms were observed in direct smears and when direct smears were negative. Since such a procedure would be particularly useful in a teaching laboratory where time is limited as well as in a diagnostic laboratory where specimens are received at various times during the day it was decided to compare this new technique with that described by McNabb<sup>2</sup> which utilizes a 3 per cent solution of hydrochloric acid in the treatment of sputum for cultivation. McNabb's technique has been used for more than ten years in this laboratory.

The use of the liquid medium described by Dubos and Davis<sup>3</sup> has been studied by a number of workers both as a medium for detecting tubercle bacilli in pathologic material and as an assay medium in tests for antibiotic activity against the bacillus. Foley<sup>4</sup> who compared fluid Dubos medium and guinea pig inoculation in the cultivation of *Mycobacterium tuberculosis* from sputum, obtained growth in the fluid medium in fifty and growth in the guinea pig in fifty four of the fifty seven sputum specimens tested. Goldie<sup>5</sup> reported cultivation of *Mycobacterium tuberculosis* in fluid Dubos medium from 116 of 400 sputum specimens. No other medium was used and guinea pigs were not inoculated by this worker. In no instance did growth fail to occur in the culture when acid fast bacilli had been observed in smear preparations of the sputum. In cultures from thirty four sputum specimens, growth in the medium was observed when no acid fast bacilli had been seen in direct smear preparations of the specimens.

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Wohnsky and Steenkin<sup>8</sup> reported fluid Dubos medium as satisfactory for testing the effect of streptomycin on *Myco tuberculosis*. Wong, Hambly, and Anderson<sup>9</sup> found Dubos-Davis medium suitable for demonstrating the antibiotic activity of subtilin against the tubercle bacillus.

For this study sputum specimens were obtained from four sanatoria and the Laboratories of the California State Department of Public Health. The sputum was homogenized and equal portions were treated with acid (McNabb's technique<sup>3</sup>) and trisodium phosphate (Corper and Stoner's technique<sup>1</sup>). Each portion of treated sputum was planted on Petriagnani medium (modification of Saenz and Constil<sup>10</sup>), and more than half of the portions were inoculated into fluid Dubos-Davis medium.<sup>4</sup> No selection of specimens to be planted in the fluid medium was made.

*A Comparison of the Effect of Treatment of Sputum With Hydrochloric Acid and Trisodium Phosphate in Preparation for Culture*—Of 245 sputum specimens treated with hydrochloric acid and trisodium phosphate (Table I),

TABLE I GROWTH OF ACID-FAST ORGANISMS ON PETRAGNANI MEDIUM FROM SPUTUM USING ACID-TREATED (MCNABB) AND PHOSPHATE-TREATED (CORPER AND STONER) PORTIONS

	NUMBER OF SPECIMENS
Growth, acid-treated portion	150
Growth, phosphate-treated portion	
No growth, acid-treated portion	66
No growth, phosphate-treated portion	
Growth, acid-treated portion	21
No growth, phosphate-treated portion	
No growth, acid-treated portion	8
Growth, phosphate-treated portion	
Total specimens	245

no growth of acid-fast bacilli on Petriagnani medium was observed from either portion of sixty-six specimens. Of the 179 specimens in which viable acid-fast bacilli were found, growth was observed on the medium inoculated with both portions of 150 sputum specimens. In twenty-one of the remaining specimens, growth of acid-fast bacilli which produced colonies typical of *Myco tuberculosis* occurred on medium inoculated with the acid-treated sputum portion, but no growth of such organisms was observed on the medium inoculated with the corresponding phosphate-treated portion. From eight specimens, growth of *Myco tuberculosis* was obtained from the portion treated with phosphate but not from the corresponding portion treated with acid. It was noted in these latter cultures that the medium was overgrown with contaminants which made the detection of rare colonies of *Myco tuberculosis* impossible.

Growth appeared earlier on the medium inoculated with acid-treated portions of the sputum specimens in 79 of the 150 specimens where growth was obtained from both portions (Table II). Since the cultures were observed only at weekly intervals, no fine differences could be seen. From sixty-five specimens, colonies were observed at the same time regardless of the method of treatment. From six specimens colonies were observed earlier on medium



inoculated with phosphate treated portions than on medium inoculated with acid treated portions. These acid treated specimens were all contaminated, which may account for the failure to discover colonies of *Mycobacterium tuberculosis*. Contaminants were completely suppressed in the cultures from the portions treated with phosphate.

TABLE II TIME OF APPEARANCE OF GROWTH FROM ACID TREATED AND PHOSPHATE TREATED SPUTUM SPECIMENS

ACID TREATED EARLIER	PHOSPHATE TREATED FASTER	SAME TIME	TOTAL
79	6	65	150

Record was made as to the relative amount of growth observed in cultures from the paired specimen portions (Table III). Only those cultures which had obviously greater amounts of growth (from two to one hundred times) were classified as greater. If the difference was less obvious the amount of growth was designated as equal. Of the 150 specimens containing viable tubercle bacilli in both portions, the amount of growth from 119 was greater in cultures from the acid treated than from the phosphate treated portion. From eighteen specimens the amount of growth was approximately the same. More growth

TABLE III AMOUNT OF GROWTH FROM ACID TREATED AND PHOSPHATE TREATED SPUTUM SPECIMENS

ACID-TREATED GREATER	PHOSPHATE TREATED GREATER	EQUAL AMOUNT	TOTAL
119	13	18	150

was noted in cultures from thirteen phosphate treated portions than in cultures from the corresponding acid treated portions. No contamination of cultures made from the phosphate treated portions was noted while in the corresponding cultures from the portion of sputum treated with acid there was considerable overgrowth by contaminants. Probably less growth of *Mycobacterium tuberculosis* was observed because of the presence of these contaminants in the cultures.

Stained preparations were made from the suspended sediment from each treated sputum portion as well as from the growth on culture medium. It was observed that acid fast bacilli in stained preparations from phosphate treated sediments or from cultures grown from phosphate treated portions in the first or second week were fragmented and distorted in morphologic appearance and irregular in staining reactions. The contrast in the appearance of organisms in stained preparations from acid treated sediments or cultures grown from acid treated portions was marked in that the organisms from the acid treated portions always appeared as nonfragmented, typical tubercle bacilli. The appearance of acid fast bacilli in stained preparations from well developed colonies after the third or fourth week of incubation did not differ when grown from acid or phosphate treated portions of sputum.

*Comparison of Growth in Dubos Davis Medium and Petragnum Medium* — Most of the Dubos Davis medium used was supplied by Difco Labora-

tones for experimental purposes 12 grams of Bacto Tb Broth Base Experimental were added to 100 ml of 5 per cent glycerol solution This mixture was sterilized at 121° C for twenty minutes To the cooled medium 10 ml of Bacto Tb Medium Serum Experimental were added aseptically and the finished product was dispensed in screw-cap Pyrex test tubes Ninety-six of the 257 plants were made in Dubos-Davis medium prepared in this laboratory according to the formula described by Dubos and Davis<sup>4</sup> Yeast autolysate, crystalline bovine albumin, and Tween 80 were used

The difference in growth in the two fluid media used is not statistically significant The Difco Experimental medium was used for the first 161 specimens and the medium made in the laboratory of the School of Public Health was used for the last ninety-six specimens The apparent superiority of this latter medium is probably due to increased facility in the preparation of smears in the later period of the study

Of the 257 (Table IV) sputum specimen portions planted on Petriagnani medium and into Dubos-Davis fluid medium, growth was observed in both media in 156 portions No growth was observed in either medium in cultures from sixty-nine sputum portions The results were in disagreement in thirty-two

TABLE IV COMPARISON OF GROWTH OF MYCO TUBERCULOSIS ON PETRAGNANI MEDIUM AND DUBOS DAVIS FLUID MEDIUM DIFCO EXPERIMENTAL AND SCHOOL OF PUBLIC HEALTH

DUBOS DAVIS MEDIUM	P GROWTH D GROWTH	P NO GROWTH D NO GROWTH	P GROWTH D NO GROWTH	P NO GROWTH D GROWTH	TOTAL
Difco experimental	93	44	17	7	161
School of public health	63	25	5	3	96
Total	156	69	22	10	257

P Petriagnani medium

D Dubos Davis fluid medium

instances Twenty-two times growth was observed on Petriagnani medium and not in Dubos-Davis fluid medium, and ten times the reverse was true Because the specimens used in the study were largely from patients with tuberculosis, the rate of agreement was so high (225 of 257) that the results could not be subject to statistical evaluation The growth of acid-fast bacilli in either of the two media under consideration was not greater than might have occurred by chance in this series of specimens

One of the advantages claimed for Dubos-Davis fluid medium is rapidity of growth which facilitates the rendering of an earlier report than is possible when solid medium is used However, if smear preparations are made from the growth on Petriagnani medium, it is possible to observe pairs or clumps of acid-fast bacilli a week or ten days before colonies are visible, and frequently a report can be made as early as is possible when fluid medium is used The technical difficulties encountered both in the making of the fluid medium and of the stained preparations of growth in the medium are greater than in the making of medium or stained preparations from the growth on Petriagnani medium The amount of time gained by cultivation in fluid medium is not great enough to compensate for the technical difficulties encountered

The effect of treatment with hydrochloric acid or trisodium phosphate as reflected in growth in fluid (Dubos Davis medium) or on solid (Petragnani) medium was observed in 127 acid treated and 130 phosphate treated portions of sputum (Table V)

TABLE V GROWTH IN DUBOS DAVIS MEDIUM AND PETRAGNANI MEDIUM INOCULATED WITH ACID TREATED AND PHOSPHATE TREATED SPUTUM PORTIONS

MEDIUM	127 ACID TREATED PORTIONS				130 PHOSPHATE TREATED PORTIONS			
	Growth	No growth	Growth	No growth	Growth	No growth	Growth	No growth
Petragnani								
Dubos Davis	Growth	No growth	No growth	Growth	Growth	No growth	No growth	Growth
Number	8	33	10	2	74	36	12	8

Growth was obtained in both media from 82 of 127 acid treated and from 74 of 130 phosphate treated portions of sputum. Neither medium revealed growth of *Mycobacterium tuberculosis* after inoculation with thirty three acid treated and thirty six phosphate treated portions. Eight specimen portions treated with trisodium phosphate grew in Dubos Davis fluid medium and not on Petragnani solid medium, whereas only two acid treated portions which grew in Dubos Davis fluid medium failed to grow on Petragnani medium. It is apparent that the bacilli surviving in the phosphate treated sputum found conditions more favorable for growth in the fluid medium. The nature of the sample (majority specimens positive) and the small number of differences involved makes a statistical evaluation of the results impossible.

#### SUMMARY AND CONCLUSIONS

Two hundred and forty five sputum specimens were homogenized and equal portions treated with hydrochloric acid (McNabb's technique) and trisodium phosphate (Corpey and Stoner's technique). Each portion was cultured on Petragnani medium and the rate and amount of growth of acid fast bacilli producing colonies recognized as typical of *Mycobacterium tuberculosis* were observed. Of 179 specimens from which growth was obtained, 150 yielded cultures from portions treated by both methods. This high proportion of agreement is a function of the weight of the excessive number of specimens containing tubercle bacilli from patients under treatment in sanatoria. (Only 74 of 245 specimens failed to show acid fast bacilli in stained preparations of the sediments used as inocula.) Divergent results were obtained with twenty nine specimens (growth from acid treated and not from phosphate treated portions in twenty one instances, and the reverse in eight instances). This difference was tested and shown to be not significant, that is the difference does not indicate the superiority of either method for the treatment of sputum in preparation for culture. The distorted appearance of the organisms in smear preparations from the phosphate treated sediments and young cultures is evidence that this treatment adversely affects the organisms. While these numbers are too small to yield statistically valid evidence the combined observations suggest that the use of trisodium phosphate tends to reduce the number of viable organisms and

to affect adversely the surviving bacilli. A sample which included a larger number of sputum specimens containing few organisms would more adequately test the techniques. The experimental evidence presented suggests that the use of trisodium phosphate for the treatment of sputum in preparation for culture may adversely affect the growth of the bacilli.

Dubos-Davis fluid medium and Petriagnani solid medium were equally effective in the cultivation of *Myco tuberculosis* from the specimen material tested in this study. The sample was so weighted in favor of positiveness that no statistically valid differences could be demonstrated. Acid-fast bacilli in sputa treated with trisodium phosphate appear to grow less readily on Petriagnani medium than the same specimens treated with hydrochloric acid. Some of these trisodium phosphate treated specimens grew in fluid medium and not on solid medium, which suggests that the fluid medium may be more satisfactory under these conditions. Because of technical difficulties in the preparation of Dubos-Davis fluid medium and in the preparation of stained smears from the growth of acid-fast bacilli in this medium, it would seem that there is little advantage in the wide-scale adoption of this medium in public health laboratories.

The technical assistance of Seiko Akahoshi, Mary Craig, Margeurite Jackson, and Joan James (senior students) is gratefully acknowledged. The collection and dispatch of specimen material used in this study by the bacteriologists in the laboratories of the State Department of Public Health and the State Sanitaria is also appreciated. The generous supply of experimental medium provided by Difco Laboratories, Detroit, Mich., is gratefully acknowledged.

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# BACTERIOPHAGE TYPING OF *SALMONELLA TYPHI*

A REPORT OF TYPING IN MICHIGAN, 1947-1948

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**B**ACTERIOPHAGE typing of *Salmonella typhi*\* has had a fairly brief but spectacular career since the development of this procedure by Craigie and collaborators in 1938. Numerous workers in various parts of the world have given it trial and have attested to its value. Nevertheless, considering its importance to the epidemiologist and to the clinician who is interested in furthering public health, the phage typing method has had only limited use. A recent canvass of public health laboratories in this country reveals that approximately seventeen institutions are employing the phage typing procedure today.

The possibility of application of the  $V_1$  phages in the treatment of typhoid fever has not been overlooked by investigators. Knorr<sup>1</sup> and associates<sup>1</sup> have used type specific phages intravenously in the treatment of fifty six patients with typhoid fever. They report that within twenty four to forty eight hours after treatment was begun patients who had been comatose regained their appetite and became cheerful and alert. Desianleau<sup>2</sup> has made use of mixtures of the four  $V_1$  phages of Craigie and Yen<sup>3</sup> for intravenous therapy.

The major use of  $V_1$  phage will undoubtedly continue to be in conjunction with the detection and identification of typhoid carriers. If the clinician who attends a sporadic case of typhoid fever as well as the epidemiologist who investigates an outbreak were to request the typing of all new strains of *S. typhi* isolated from either cases or suspected carriers, the beneficial results would soon be evident to both the physician and the public health authorities. Should a new carrier be discovered for even a part of the sporadic cases that occur, the total elimination of the disease would be much hastened. For it is evident that the known carrier who is excluded from food handling occupations and who is aware of the possibility of infecting others is seldom a hazard.

The initial discovery that led to typing was the finding by Craigie and Brandon<sup>4</sup> of certain strains of bacteriophages which would lyse typhoid bacteria bearing the  $V_1$  or so called virulence antigen. Four  $V_1$  specific phages were isolated by Craigie and Yen<sup>5</sup>, one of which had the peculiar ability to adapt itself to certain strain differences of cultures of *S. typhi* containing  $V_1$  antigen. These strain differences were not demonstrable by any means other than  $V_1$  phage action. By adaptation and selective propagation of variants of this unusual phage, eleven type specific phages were produced. These phages when applied to numerous strains of typhoid bacteria isolated from a variety of cases and localities demonstrated that the types of *S. typhi* were limited in number and were readily differentiated.

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The nomenclature used in this paper is that of the Kauffmann-White schema.

It was further observed that there was a notable agreement of the results obtained in the laboratory with epidemiologic data.<sup>6</sup> For example, the recovery of the same phage type from a carrier and a group of connected cases was found to agree with the results of field investigations. On the other hand, the recovery of dissimilar types from a carrier and a group of related cases was found sufficient grounds for excluding the carrier from consideration as the source of the outbreak. In the large majority of such cases further investigation revealed the carrier responsible for the outbreak.

Of first importance is the fact that phage types of *S. typhi* have, with only a few rare exceptions, been found to remain stable both in vivo and in vitro. Thus it is possible to determine the phage type of the typhoid organism which a carrier is excreting, with the expectation that the type will not change either in the original or in a new host.

New phage types of *S. typhi* have been recognized since the original work of Craigie and Yen and today there are twenty-five known types. The typing technique,<sup>3, 13</sup> however, is relatively unchanged from the method worked out in 1938.

#### OBSERVATIONS

Previous to 1948, the Michigan Department of Health investigated two outbreaks of typhoid fever in which bacteriophage typing contributed necessary evidence for indication of responsible carriers.\* Because of these experiences in which the value of typing was clearly shown, the technique was instituted in our laboratories as a trial procedure in October, 1947. Over a period of one year the method of Craigie and Yen<sup>3</sup> was applied to 432 cultures of *S. typhi* which were isolated from 209 individuals, representing both cases of typhoid fever and carriers.

Table I shows the various types identified during the period October, 1947, to October, 1948. Of the 432 cultures, we were able to type 341 (78.90 per cent),

TABLE I VI PHAGE TYPES OF *SALMONELLA TYPHI* ISOLATED FROM CASES AND CARRIERS IN MICHIGAN—OCTOBER, 1947, TO OCTOBER, 1948

TYPE	CASES		CARRIERS		TOTAL			
	IND *	ISOL †	IND	ISOL	IND	%	ISOL	%
A	16	32	17	31	33	15.80	63	14.60
B <sub>1</sub>	0	0	1	3	1	0.48	3	0.69
B	3	7	3	5	6	2.87	12	2.78
B <sub>2</sub>	2	3	2	5	4	1.91	8	1.85
C	7	8	15	36	22	10.50	44	10.20
D <sub>1</sub>	6	11	5	10	11	5.26	21	4.86
D <sub>1</sub> HP <sub>10</sub>	2	2	5	8	7	3.35	10	2.32
D	1	2	1	2	2	0.96	4	0.92
D <sub>2</sub>	0	0	2	2	2	0.96	2	0.46
E <sub>1</sub>	44	91	26	54	70	33.45	145	33.60
F <sub>1</sub>	4	8	8	17	12	5.75	25	5.79
J	2	3	0	0	2	0.96	3	0.69
N	0	0	1	1	1	0.48	1	0.23
Untypeable	17	41	19	50	36	17.20	91	21.10
Total	104	208	105	224	209		432	

\*Individuals  
†Isolations

\*The typing of strains from both outbreaks was carried out by courtesy of Dr. James Craigie.

while 91 (21.10 per cent) proved to be untypable. Our experience with untypable strains is evidently the same as that of many other workers. Buckle<sup>7</sup> in his comprehensive review of typing all over the world, reports that 21.96 per cent of typhoid strains were found by various people to be untypable.

During the period covered by this report there were 83 reported cases of typhoid fever in Michigan from which we received 208 cultures for typing. Positive typings were obtained on 167 (80.28 per cent). Cultures from typhoid carriers, for the same period totaled 224 of which 174 (77.70 per cent) were typed. Among the 50 untypable cultures isolated from carrier specimens were seven strains of a small colony variant of *S. typhi* which appeared to be similar to those described by Morris and co-workers.<sup>8,9</sup> The various small colony strains were all isolated from repeat specimens from the same carrier and proved to be W in form.

The distribution of types found in Michigan is comparable for the most part to that reported by authors in other parts of the world.<sup>7</sup> Phage type E<sub>1</sub> predominated in our series making up 33.60 per cent of the total cultures received. Included in the 145 type E<sub>1</sub> strains are cultures isolated from two outbreaks.

In September, 1947, cases of typhoid fever were reported from a limited area on the outskirts of one of Michigan's larger cities. Investigation showed that a factor common to all patients was the drinking water which came from a neighbor's well. Bacteriophage typing confirmed the fact that all cases were probably infected from a single source, since all cases yielded cultures which were phage type E<sub>1</sub>. It was discovered that the owner of the well had recently installed running water in his house and at the same time had provided a new bathroom and septic tank. The disposal field for the septic tank was located quite close to the well, and once the tank was in use cases of typhoid fever began to appear in the neighborhood.

Members of the family owning the well were not affected, probably because they had discontinued use of the well water. Within a comparatively short time there were fourteen cases of typhoid. Specimens from the four members of the family were examined and two were found positive for *S. typhi*. It was soon proved that Grandmother S. was a typhoid carrier excreting phage type E<sub>1</sub> organisms and that her granddaughter was a probable contact carrier. The granddaughter like her grandmother excreted phage type E<sub>1</sub> organisms.

The second type E<sub>1</sub> outbreak involved persons eating in a factory cafeteria. Epidemiologic evidence was again supported by phage typing. Specimens from seven patients contained cultures of *S. typhi* which were all phage type E<sub>1</sub>. Investigation of the food handlers revealed a typhoid carrier who was excreting phage type E<sub>1</sub>. The closing of the cafeteria resulted in the cessation of the outbreak.

Other than these two epidemics the remainder of the 208 case cultures were obtained from sporadic cases of typhoid fever. Bradley<sup>10</sup> refers to these sporadic cases as "chinks in the complex armor of modern sanitation" and states further that "it is only by counting these sporadic cases which fail to initiate outbreaks that we have a true measure of the efficiency of our systems."

It will be noted from Table I that 10 strains of phage type D<sub>4</sub>HP<sub>56</sub> were identified. Phage D<sub>4</sub>HP<sub>56</sub> is the newest of typing phages and was produced by Craigie<sup>11</sup> from cultures submitted by the Michigan Department of Health following an outbreak in Highland Park, Mich., in the summer of 1946. A detailed report of this outbreak is in preparation.<sup>12</sup> As far as we are aware, the isolation of D<sub>4</sub>HP<sub>56</sub> has not been reported elsewhere in the literature.

Our typing experiences have included numerous opportunities to examine repeat cultures from several individuals. We have typed successfully thirteen cultures isolated from each of two individuals, and in one case we have received fifteen cultures of the same type from one person. With but one exception, repeat specimens have all yielded the original type. The exception was a carrier who was apparently excreting two phage types. A discussion of this carrier will be presented in a forthcoming paper.<sup>12</sup>

At the beginning of this study it was our intention to conduct a survey of the 266 known carriers in Michigan for the purpose of indicating the phage type, where possible, to the Bureau of Disease Control. The desirability of having all known carriers typed has been stressed by numerous workers in this field, among whom are Craigie<sup>13</sup> and Morris and associates.<sup>14</sup> Our original intention has been only partially fulfilled, chiefly because cultures have been submitted from only 105 carriers. However, there is some doubt in our minds about the value of a complete typing on all carriers in Michigan, at least from the standpoint of epidemiologic control. It has been our experience that we have not received a culture from a case of typhoid fever who was infected by a carrier known to the Bureau of Disease Control. This fact was confirmed by the Director of the Bureau who stated that there have been only three cases of typhoid fever traceable to known carriers since the carrier control program was begun in 1932.<sup>15</sup>

Korns and Trussell<sup>16</sup> state that at the time of their writing 465 known chronic carriers were listed in New York State. It is estimated that 2,500 carriers are included in New York's population. It seems probable that a similar condition exists in Michigan. However, in the vast majority of cases, the carrier will be an unknown. In our limited experience the indications are that phage typing will be of value in this state, especially for identification of the true carrier in sporadic cases and outbreaks of typhoid fever.

#### SUMMARY

1. Bacteriophage typing has been applied to 432 cultures of *S. typhi* isolated in Michigan. Positive typings were obtained with 341 cultures representing thirteen different phage types. Among the 341 cultures were 167 from active cases of typhoid, while 174 cultures were typed from carriers. Eleven phage types were found among the case cultures and twelve were demonstrated from carrier cultures.

2. Ten strains of the new phage type D<sub>4</sub>HP<sub>56</sub> were identified.

3. Two epidemics, in which phage typing was of value in arriving at a solution, are discussed.



4 Attention is drawn to the fact that there have been only three cases of typhoid fever in Michigan that were traced to known typhoid carriers since 1932. It is felt that the known typhoid carrier is seldom a public health menace.

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## FORMATION OF ANTIBODIES IN HUMAN SUBJECTS AFTER THE INGESTION OF HEAT-KILLED BRUCELLA ABORTUS

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THE agglutination test is widely used in the diagnosis of brucellosis. Results must be interpreted cautiously, however, because agglutinins are known to appear in the sera of persons who do not have brucellosis. Those conditions other than active brucellosis in which serum agglutinins for *Brucella* may be present are shown in Table I. The maximum titers which have been observed are also listed. After therapy with streptomycin and sulfadiazine, there is a persistent elevation of titer for as long as a year following complete remission in some patients.<sup>1</sup> These individuals have been checked carefully at the University of Minnesota Hospitals for evidence of continued bacteremia as well as clinical signs of infection, and neither has been found. It is well known that the introduction of *Brucella* antigens into the tissues, either intradermally for diagnostic purposes or for therapeutic ends, stimulates the production of agglutinins.<sup>2</sup> Similar inoculation with cholera antigens has the same effect according to the work of Eisele, McCullough, and Beal.<sup>3</sup> Other conditions in which cross agglutinins for *Brucella* occur are tularemia and typhoid fever.<sup>4</sup> In addition, it has been suggested that the titer of *Brucella* agglutinins may rise in response to an anamnestic reaction stimulated by another infection. Boits<sup>5</sup> mentions a rise to 1:160 occurring in pneumococcal pneumonia.

These examples probably account for only a small percentage of what might be called "false-positive reactions." At the University of Minnesota Hospitals, Aagaard<sup>6</sup> has observed that *Brucella* agglutinins were present in 93 per cent of blood samples obtained from 352 consecutive rural patients who were being studied in a dispensary service for various causes. A diagnosis of brucellosis was established in only one of these patients. The present investigation was undertaken to determine whether the agglutinins demonstrated in the sera of these patients could have been produced by the repeated ingestion of pasteurized milk containing large numbers of nonviable *Br. abortus*. Dolman, Hudson, and Mathias<sup>7</sup> studied the sera of healthy volunteers who drank suspensions containing about 210 billion *Br. abortus* in divided doses over a period of six weeks. They found that no rise in serum agglutinins resulted and they concluded that it was unlikely that a titer of 1:20 or higher could be provoked in man by the ingestion of milk containing dead *Brucella* organisms. Only eight persons were included in their experiment.

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TABLE I. CONDITIONS OTHER THAN ACTIVE BRUCELLOSIS IN WHICH SERUM AGGLUTININS FOR *BRUCELLA* MAY BE PRESENT (TITER OF AGGLUTININS)

Following successful anti-brucella therapy	1 2,560
After injection of Brucella antigens for skin test or vaccine therapy	1 1,280
After cholera vaccination	1 2,560
Tularemia	Low
Typhoid fever	1 160
Anamnestic reactions	1 160

## METHODS

*Br abortus* strain 1257 obtained from the National Institute of Health, was used throughout this study. Cultures grown for forty eight hours on tryptose phosphate agar were suspended in physiologic saline solution and were killed by heating at 65° for thirty minutes. The suspensions prepared in this manner were carefully checked for sterility and phenol was added in a concentration of 0.5 per cent. Appropriate concentrations of bacteria were made by adjusting the density of the suspensions in a photoelectric colorimeter. The colorimeter had been standardized at a density of 1 million *Brucella* cells per milliliter by means of a suspension of living organisms whose colony count had been determined by the standard pour plate method. Guinea pigs were selected as the initial subjects because of the demonstration by Ratner and Gruelke that undigested antigens are absorbed through the normal gastrointestinal wall of these animals at all ages. Healthy guinea pigs which had been obtained from reliable sources and which weighed approximately 400 grams were used. Water was withheld from their diet during the experimental period as it was found that animals which were not thirsty failed to swallow the *Brucella* suspensions. The aqueous suspensions were fed to guinea pigs by inserting the tip of a tuberculin syringe into their mouths and slowly injecting a measured volume. The animals were divided into two groups as shown in Table II. One group received a single feeding of 100 billion organisms. The other group received the same amount in ten divided feedings over a period of fifteen days. Blood was drawn for agglutination tests by cardiac puncture immediately preceding the period of feeding and also three and five weeks after this period was started.

TABLE II. FAILURE TO INDUCE THE FORMATION OF AGGLUTININS IN GUINEA PIGS FED HEAT KILLED *Br abortus*

NUMBER OF GUINEA PIGS	NUMBER OF ORGANISMS PER FEEDING	NUMBER OF FEEDINGS	NUMBER DEVELOPING AGGLUTININS
9	100 billion	1	0
8	10 billion	10	0
Total 17			0

The effect of ingestion of these organisms was also studied in human volunteers. A summary of the procedure which was used for feeding these persons is given in Table III. The first group was composed in part of medical students in apparent good health. The remainder were young men who had recovered from acute polyomyelitis and were hospitalized in a convalescent home. The general condition of these patients was excellent. The subjects in Groups 2 and 3 were hospital patients. The vast majority of these were hospitalized for

TABLE III. METHODS OF ADMINISTERING HEAT KILLED *BRUCELLA* TO THREE GROUPS OF HUMAN SUBJECTS

GROUP	NUMBER OF SUBJECTS	NUMBER OF ORGANISMS	VEHICLE	NUMBER OF FEEDINGS	LENGTH OF FEEDING PERIOD
1	27	100 billion	Water	1	
2	76	100 billion	Pasteurized milk	10-20	10-20 days
3	8	1 billion	Pasteurized milk	10-12	12-20 days

plastic surgery or treatment of fractures Organic disease of the gastrointestinal tract was demonstrated in only four of the sixty seven individuals Those in Group 1 received a single feeding of 100 billion *Brucella* in water Preliminary studies were made with this large dose to determine whether there was any possibility of producing agglutinins by the ingestion of *Brucella*

In the case of Groups 2 and 3, an attempt was made to reproduce the naturally occurring circumstances in which heat killed *Brucella* may be ingested in pasteurized milk This was done by the addition of the *Brucella* suspension once a day to milk which the patients consumed in their regular hospital diet This milk was furnished to the University Hospital by the University of Minnesota School of Dairy Husbandry where it is pasteurized and bottled Samples were tested for whey agglutinins on several occasions and the titers obtained were found to range from 0 to 1:10 By far the greatest proportion of this pooled milk came from *Brucella* free herds The small percentage of milk coming from animals infected with brucellosis was so greatly diluted that the final pasteurized product which was ingested by the hospital patients can be considered free of all but negligible amounts of *Brucella* antigen or antibody

Members of Group 2 received 100 million organisms daily in this fashion for ten to twenty feedings during periods of ten to thirty days The dose was reduced to a million organisms daily in Group 3 to determine whether a relatively small number of cells might be effective Blood was collected when the feedings were commenced and two to three weeks thereafter Agglutination tests were performed on each sample of blood by the standard tube method\* *Brucella* opsonocytophagic tests were performed on sixteen subjects by the method of Jersild<sup>9</sup> These were interpreted on the basis of the Foshay nomogram<sup>10</sup>

#### RESULTS

Agglutinins failed to appear in the sera of any of the seventeen guinea pigs (Table II) Agglutinin production occurred in human subjects, however, in each of the three categories (Table IV) Of the twenty-three subjects who were fed a massive number of heat killed *Brucella*, four developed agglutinins This indicated the feasibility of experimenting with smaller numbers, and it was found that after the repeated ingestion of 100 million cells, significant titers of agglutinins were detected in eleven of the thirty-six persons When the total number was reduced to 1 million daily, one of eight additional patients showed a rise in titer Of the entire total of sixty-seven individuals, agglutinins were produced in sixteen or 23.5 per cent

In Tables V, VI, and VII there is an analysis of the individual agglutinin responses Surprisingly high titers developed in group 1 One individual in this group displayed a rise in titer from 0 to 1:640, two to 1:160, and one from 1:20 to 1:80 The last-mentioned change was significant because there was not only a four-fold rise in titer but also a striking change in the phagocytic index

TABLE IV AGGLUTININ FORMATION FOLLOWING THE INGESTION BY HUMAN SUBJECTS OF HEAT KILLED *BR.* ABORTUS

GROUP	NUMBER OF SUBJECTS	NUMBER OF ORGANISMS PER FEEDING	NUMBER OF FEEDINGS	NUMBER DEVELOPING AGGLUTININS
1	23	100 billion	1	4 (17.0%)
2	36	100 million	10-20	11 (30.5%)
3	8	1 million	10-12	1 (12.5%)
Total	67			16 (23.9%)

\*The tube antigen was prepared by the Bureau of Animal Industry, Beltsville, Md.

TABLE V SUBJECTS IN WHOM SERUM AGGLOUTININS DEVELOPED AFTER A SINGLE ORAL ADMINISTRATION OF 100 BILLION HEAT KILLED BRUCELLA ORGANISMS IN AQUEOUS SUSPENSION

GROUP 1	SEX	AGE	INITIAL TITER	TITER AFTER FEEDING
SUBJECT				
A S	M	45	0	1 160
D Y	M	18	1 20	1 80
B A	M	12	0	1 640
D O	M	26	0	1 160

TABLE VI SUBJECTS IN WHOM SERUM AGGLOUTININS DEVELOPED AFTER DAILY FEEDINGS OF ONE HUNDRED MILLION HEAT KILLED BRUCELLA CELLS

GROUP 2	SEX	AGE	INITIAL TITER	TITER AFTER FEEDING
SUBJECT				
I A	F	61	0	1 40
E P	F	70	0	1 80
B L	M	88	0	1 40
O M	F	73	0	1 20
O L	F	58	0	1 20
J S	M	66	0	1 160
C H	M	62	0	1 40
O N	F	54	0	1 40
N L	M	61	0	1 40
J P	M	48	0	1 20
F W	M	60	0	1 160

TABLE VII AGGLOUTININ FORMATION FOLLOWING THE DAILY INGESTION OF ONE MILLION HEAT KILLED BR ABORTUS

GROUP 3	SEX	AGE	NUMBER OF FEEDINGS	INITIAL TITER	TITER AFTER FEEDING
SUBJECT					
A T	M	41	10	160	80
S P	F	80	10	0	0
E B	F	29	10	0	0
C R	M	59	10	0	0
O S	M	68	10	0	0
L N	M	58	12	80	±40
A O	M	49	10	0	0
B S	M	56	12	0	40

TABLE VIII PHAGOCYTIC INDICES BEFORE AND AFTER THE INGESTION OF HEAT KILLED BR ABORTUS IN SUBJECTS WHOSE SERA DISPLAYED A RISE IN AGGLOUTININ TITER

SUBJECT	INITIAL AGGLOUTININ TITER	AGGLOUTININ TITER AFTER FEEDING	INITIAL PHAGOCYTIC INDEX	PHAGOCYTIC INDEX AFTER FEEDING
A S	0	160	2	92
B A	0	640	3	100
D O	0	160	4	92
D Y	20	80	12	98
F W	0	160	1	11
I A	0	40	7	1
E P	0	80	5	8
B L	0	40	27	16
O M	0	20	5	5
O L	0	20	9	7
J S	0	160	5	91
C H	0	40	7	7
O N	0	40	7	19
N L	0	40	4	6
J I	0	20	2	2
B S	0	40	2	4

(Subject D Y, Table VIII) The titers developed by the next group (Group 2) were lower and ranged from 1:20 to 1:160. In Group 3, the single elevation which occurred was to 1:40.

The sera of the sixteen subjects in whom agglutinins for *Brucella* had appeared were also tested for opsonins before and after the ingestion of *Brucella*. In seven of these there were increases ranging from 10 to 90 over the initial phagocytic index (Table VIII).

Finally, the ability of dead *Brucella* to sensitize the skin was investigated in eighteen persons who ingested 100 million organisms daily for ten to twenty feedings. Sixteen of the eighteen had negative skin tests with *Brucelle*igen antigen after the feedings were discontinued. Two persons having positive tests at the beginning of the experiment showed no alteration in skin sensitivity as a result of the feedings.

#### DISCUSSION

These results demonstrate that the ingestion of nonviable *Brucella* may give rise to the production of agglutinins or opsonins. This may help explain the occurrence of agglutinins in the blood of some asymptomatic persons who inhabit areas where Bang's disease is prevalent in cattle. Under naturally occurring circumstances, dead *Brucella* may be ingested in pasteurized milk or may result from the action of gastric juice on living organisms which enter the stomach in raw milk. Studies now in progress in this laboratory have demonstrated a definite bactericidal action of human gastric juice against *Brucella*.

This study also suggests that the development of dermal hypersensitivity does not accompany the production of agglutinins after the ingestion of dead *Brucella*. Most individuals whose sera contain *Brucella* agglutinins also display dermal hypersensitivity to *Brucella* antigens. In the survey made by Aagaard,<sup>6</sup> the incidence of negative intradermal reactions was only 4 per cent in such persons. In a group of sixty-five subjects giving positive agglutination tests, Braude<sup>2</sup> found only four (6 per cent) who did not react to any one of four *Brucella* antigens applied intradermally. Persons who might acquire agglutinins in their sera by the ingestion of pasteurized milk containing large numbers of heat-killed *Brucella* would, therefore, be expected to comprise a relatively small percentage of all those having positive agglutination tests.

Several factors may account for the negative intradermal reactions which were observed in subjects who were examined for hypersensitivity after ingesting heat-killed *Brucella*. The quantity of *Brucelle*igen employed in each test was the usual dose of 0.1 milligram. This amount may be inadequate for eliciting positive reactions in the presence of a small degree of hypersensitivity. Exposure of the organism to both heat and gastric juice may remove the antigenic components which are responsible for inducing hypersensitivity. It has been reported that dead *Brucella* may induce hypersensitivity when injected into the tissues of experimental animals. Leon and Sosa<sup>11</sup> observed that positive intradermal reactions to *Brucella* antigens were obtained in rabbits after the repeated injection of dead *Brucella*. Similar results have been noted in this laboratory in studies made in guinea pigs, although the reactions were of much weaker

intensity than those occurring in animals infected with *Br abortus*. On the other hand, Fleischner and Meyer<sup>1</sup> were able to stimulate the production of agglutinins in the sera of guinea pigs but could not sensitize their skins by the repeated injection of millions of heat killed Brucella. Stroem<sup>13</sup> failed to produce sensitivity in guinea pigs when he killed Brucella were introduced with an adjuvant (Kieschulte). These discrepancies in the reports of various authors may be the result of differences in the strains and quantities of Brucella which were used in the respective experiments. In the present investigation, observations were limited to the effects of only one strain. It will be necessary to repeat these studies in human subjects with a number of strains administered over a longer period of time.

It is presumed that the production of agglutinins in this study was related to the passage of Brucella antigen from the intestine into the blood. Such an absorption of undigested antigens by the intestinal mucosa has been demonstrated by several investigators. By means of the Prausnitz-Kustner reaction the absorption of undigested fish and egg proteins in human beings has been noted by Walzer<sup>14</sup> and Ratner and Gruchl.<sup>8</sup> They have also shown that guinea pigs may be sensitized and later killed by anaphylactic shock if they are fed cows' milk on successive occasions. Hektoen<sup>7</sup> using the precipitin test, detected the presence of thyroglobulin in the portal blood of dogs which were fed beef thyroid. The results which we obtained by feeding dead Brucella organisms may be regarded as additional evidence of the permeability of the intestinal mucosa to proteins. There is also a possibility that the whole Brucella cell can permeate the intact mucosa. Arnold<sup>15</sup> has found that typhoid and coliform bacilli entered the thoracic duct and reached the liver in large numbers after these bacilli were placed in the duodenum of dogs.

It is difficult to explain why agglutinin failed to appear in the sera of guinea pigs after they were fed massive doses of heat killed Brucella. One of us (A. B.) has observed in other experiments that agglutinins can be produced in guinea pigs by the injection of heat killed *Br abortus* into their skins. The titers which were produced in the sera of these animals were somewhat less than might be expected. Animals which were injected with a total of one billion to ten billion organisms over a period of eight to twelve weeks developed agglutinin titers in their sera which seldom exceeded 1:160 and often measured only 1:40 or 1:80. It appears therefore that agglutinins may not be produced readily in guinea pigs by the administration of dead Brucella and that oral doses even more massive than we employed may be necessary for the production of agglutinins in these animals.

#### SUMMARY

1. Heat killed Brucella cells were suspended in water or in milk and fed to sixty-seven human volunteers. In sixteen, or 23.9 per cent of the individuals, agglutinins appeared in the sera after the period of feeding.

2. Dermal hypersensitivity to Brucella antigen was not induced by the ingestion of nonviable Brucella.

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## LABORATORY AND CLINICAL OBSERVATIONS ON AEROSPORIN (POLYMYXIN B)

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**A**N ANTIBIOTIC substance with remarkable activity against gram negative bacteria was reported recently by Brownlee and Bushby.<sup>1</sup> This substance, Aerosporin (Polymyxin A) is a polypeptide produced by the soil organism *Bacillus aerospirius* Green. The bacillus was found to be identical with *Bacillus polymyxa* (Prazmowski) Migula<sup>2</sup> from which Stansky and co-workers<sup>4</sup> had derived another antibiotic substance Polymyxin (Polymyxin D). Further study indicated<sup>3</sup> that *B. polymyxa* produced a number of peptides with similar antibiotic properties but differing from each other in their amino acid content and their toxicity for animals.

One of the polypeptides (Polymyxin D), extensively investigated in the laboratory by Stansky and associates<sup>4,5</sup> was given an early clinical trial by the group at the Johns Hopkins Hospital.<sup>6,11</sup> Polymyxin D was very active against many gram negative bacteria both in vitro and in animals, its toxicity for animals was relatively low and it appeared to be clinically useful. However, definite evidence of renal toxicity was soon discovered and clinical use of the drug was abandoned.<sup>12</sup>

Another peptide produced by *B. polymyxa*, Aerosporin (Polymyxin A) was shown to be five to fifty times more active by weight against certain gram negative bacteria than streptomycin.<sup>1</sup> Its antibacterial spectrum resembled that of Polymyxin D. It was claimed to be bactericidal rather than bacteriostatic and susceptible organisms did not readily develop resistance to its action. It did not contain the amino acid, *D* serine believed to be nephrotoxic. Clinical trials<sup>13</sup> of Polymyxin A indicated good response in children with whooping cough and in other infections with gram negative bacteria.

These reports suggested a trial of this group of drugs in infection of the urinary tract caused by gram negative organisms in which the effect of the antibiotic could be studied quantitatively by bacteriologic methods. Observations made in the laboratory and on patients in the course of these trials are reported here.

### MATERIALS AND METHODS

The drug preparation used was Aerosporin Brand Polymyxin B (Hydrochloride) ND No 110,\* hereafter referred to as Polymyxin B. It was supplied

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in vials each of which contained 60 mg of white amorphous material equivalent to 40 mg of Polymyxin B. Solutions of desired strength were prepared by dissolving the contents of a vial in unbuffered 0.85 per cent sodium chloride solution. At the beginning of this investigation a random sample of Polymyxin B was diluted to a concentration of 200  $\mu$ g per milliliter, frozen, and stored in ampules at  $-70^{\circ}\text{C}$ . This sample was used as standard of reference in all laboratory tests.

Bacteria isolated from the urine of patients with infection of the urinary tract were grown in Proteose-peptone broth (Difco) for eighteen hours at  $37^{\circ}\text{C}$  and the density of the culture was then adjusted with broth to that of the McFarland tube No. 2. For the determination of bacterial sensitivity, 0.2 ml of a  $10^{-4}$  dilution of such a culture was used in a tube dilution procedure slightly modified from Brownlee and Bushby<sup>1,5</sup>. The tubes were uniformly incubated at  $37^{\circ}\text{C}$  and read after eighteen hours. A standard organism (*Escherichia coli*) of known and constant susceptibility to Polymyxin B was included in each test.

Serum levels of Polymyxin B were estimated by a method<sup>5</sup> similar to that used for determination of penicillin in blood serum<sup>14</sup>. The frozen stock of Polymyxin B served as standard of reference.

#### EXPERIMENTAL

*1 Physical Characteristics of the Drug*—The contents of vials dissolved rapidly and completely in water or physiologic salt solution, forming a clear, colorless solution of pH 7.2 to 7.4. Sixty milligrams dissolved readily in 2 ml of solvent. The stability of such solutions was remarkable. At  $-70^{\circ}\text{C}$ , in solutions containing 200  $\mu$ g per milliliter, there was no loss of activity over a period of five months. Activity was evaluated both against organisms of known and stable sensitivity and against freshly prepared solutions. Although very dilute solutions deteriorated slowly at room temperature, in concentrations of 4 mg of Polymyxin B per milliliter antibacterial activity was not significantly diminished after sixty-two days at either  $4^{\circ}\text{C}$  or  $37^{\circ}\text{C}$ . The dry preparation was stable for at least five months at  $22^{\circ}\text{C}$ .

Changes in hydrogen ion concentration did not greatly influence the antibacterial effect of the drug. Polymyxin B had the same effect on the standard strain of *Esch. coli* when tested in buffered broth at pH 4.2, 5.0, 7.0, or 7.4 and was only slightly less inhibitory at pH 9.2.

*2 Sensitivity to Polymyxin B of Bacteria Isolated From the Urine*—Strains of bacteria freshly isolated from the urine of patients were examined for their sensitivity to Polymyxin B in vitro. Table I indicates the range of organisms isolated and their relative sensitivities. The activity of Polymyxin B against bacteria encountered frequently in urinary tract infections agrees well with that observed for Polymyxin D<sup>7</sup>. Early clinical experience indicated that organisms with a sensitivity of 1.0  $\mu$ g per milliliter or more in vitro uniformly failed to respond to therapy with Polymyxin B. Determinations of sensitivity were consequently limited to a range 0.04 to 1  $\mu$ g per milliliter.

TABLE I IN VITRO SENSITIVITY TO POLYMYXIN B OF BACTERIA ISOLATED FROM URINARY TRACT INFECTIONS

BACTERIAL SPECIES	NUMBER OF STRAINS	NUMBER NOT IN HIBITED BY $\mu\text{g./ML}$	NUMBER OF STRAINS INHIBITED BY POLYMYXIN B				
			$\mu\text{g./ML}$				
			1	0.4	0.2	0.1	0.05
Escherichia coli	23	0	0	2	15	4	1
Aerobacter aerogenes	16	0	6	8	2	0	0
Pseudomonas aeruginosa	18	4	5	8	1	0	0
Proteus vulgaris	5	3	2	0	0	0	0
Streptococcus faecalis	7	7	0	0	0	0	0
Shigella sp	4	2	1	0	1	0	0
Salmonella sp	4	0	0	2	1	1	0
Paracolon bacilli	2	0	0	0	2	0	0

All strains of *Esch coli*, *Salmonella* and *paracolon bacilli* which were examined were inhibited by  $0.4 \mu\text{g}$  per milliliter or less. *Aerobacter aerogenes* strains tended to be less susceptible, and *Proteus vulgaris* and *Pseudomonas aeruginosa* were still more resistant. This scale of susceptibility resembles that commonly experienced with streptomycin and ampicillin.<sup>15</sup>

The action of Polymyxin B appeared to be bactericidal rather than bacteriostatic. The end point in a tube dilution series was usually not changed when the test was incubated for seven days at  $37^{\circ}\text{C}$  after the first observation. There was no growth in subcultures made on solid or in liquid media from the last tube in which no growth occurred. The growth of resistant strains in the presence of Polymyxin B did not destroy it. These organisms, grown in broth containing the drug were centrifuged out and the supernatant fluid was tested for antibiotic activity with the standard strain of *Esch coli*. Its activity was unchanged.

**3 Bacterial Resistance to Polymyxin B**—An effort was made to induce resistance to Polymyxin B in vitro with four susceptible bacterial strains. Daily transfers were made in broth containing increasing amounts of the antibiotic. The increase in resistance in these four random gram negative organisms is shown in Fig 1. The strain of *A. aerogenes* was originally inhibited by  $0.2 \mu\text{g}$  per milliliter and after a regular step wise increase in resistance in the course of thirteen transfers it grew freely in  $1,000 \mu\text{g}$  per milliliter. A strain of *Ps. aeruginosa* showed a similar but less regular change in susceptibility. One strain each of *Esch coli* and *Salmonella choleraesuis* changed but little in two weeks of daily transfers in broth containing Polymyxin B. Not only did these organisms fail to increase in resistance but also repeatedly, no growth was obtained when subcultures were made from tubes in which there was visible turbidity perhaps indicating that the drug was slowly killing the organism.

Strains that acquired resistance to Polymyxin B kept this characteristic when subcultured daily for fourteen days in plain broth. No bacteria dependent on Aerosporin (Polymyxin B) for growth have been observed to date.

The occurrence of naturally resistant individuals in bacterial populations was tested by means of massive inocula on solid media. When Polymyxin B was added to Proteose peptone agar (Difco), tests made with small inocula of bacteria indicated sensitivities comparable with those found by the tube

TABLE IV TREATMENT OF A PATIENT WITH URINARY TRACT INFECTION WITH POLYMYXIN B  
RELATION OF DRUG TO CLINICAL AND LABORATORY FINDINGS  
(A 73 YEAR OLD MAN WHO HAD A NEPHRECTOMY TEN YEARS BEFORE, NOW HAS PYELONEPHRITIS  
[CHRONIC] IN THE REMAINING KIDNEY)

Day	1	2	3	4	5	6	7	13	27
Polymyxin B	0	120	80	120	80	0	0	0	0
Dose (mg/day)									
Average serum levels ( $\mu\text{g/ml}$ )	0	13	25	410	410	14	0	0	-
Toxic symptoms									
Paresthesias	0	+	++	+	+	-	-	-	-
"Dizziness"	0	-	+	+	+++	+	-	-	-
Ataxia	0	-	-	+	-	-	-	-	-
Blood examinations									
Hemoglobin (Gm %)	15.0					15.3			14.8
White blood cells/ $\text{c mm}$	6,000					8,750			9,500
Polymorphonuclears (%)	65					69			71
Eosinophiles (%)	1					4			2
Blood urea (mg %)	72					51			59
Urea nitrogen (mg %)	34					21			24
Urine examinations									
White cells/high dry field	++++	+++	+++	++	510	23	23	23	++
Red cells/high dry field	+	+	+	Rare	Rare	0	0	0	Occasional
Bacteria/high dry field	++++	+++	++	+	Rare	Rare	0	0	+++
Protein (mg %)	52	21			63		25		44
Urine culture (Bacteria/ ml)									
Esch coli	80,000		1,000		0	0	0	0	20,000
Proteus sp	1,000		100		10		10	50	500

they were resumed. No effect was observed on the red or white cell count in the ten patients.

The most marked undesirable effect of Polymyxin B was on the central nervous system. Within forty-eight hours of inception of treatment, the majority of patients experienced circumoral paresthesias and "dizziness" without nausea, anorexia, or true vertigo. There was commonly mild ataxia and occasionally the feeling of weakness of the legs without objective evidence of diminished muscular strength. These symptoms lasted while the drug was administered and subsided in all patients in thirty-six to forty-eight hours after it was discontinued. No residual disturbances were observed in any patient.

With doses larger than those routinely used, more drastic effects on the central nervous system were observed. Two patients received 160 mg of Polymyxin B intramuscularly in twelve hours (i.e., 25 mg per kilogram per twelve hours). They experienced peripheral as well as circumoral paresthesias, severe incoordination, with dysarthria, dyssynergia, dysmetria, ataxia, a positive Romberg sign and marked past pointing. The reflexes were within physiologic limits. There was no true vertigo, only "lightheadedness," no tinnitus or impaired hearing, and the appetite was unimpaired. These symptoms and signs persisted for thirty-six hours while the serum concentration of the drug was in excess of 10  $\mu\text{g}$  per milliliter; they then gradually subsided and left no sequelae.

An effort also was made to find out whether exposure of bacterial populations to Polymyxin B for three to five days *in vivo* might induce resistance. In a number of patients the sensitivity of strains recovered prior to treatment could be compared with that of similar organisms cultured from the urine after therapy. No increase in resistance was observed, regardless either of the clinical course or of the response to the drug.

## SERUM LEVELS OF POLYMYXIN B

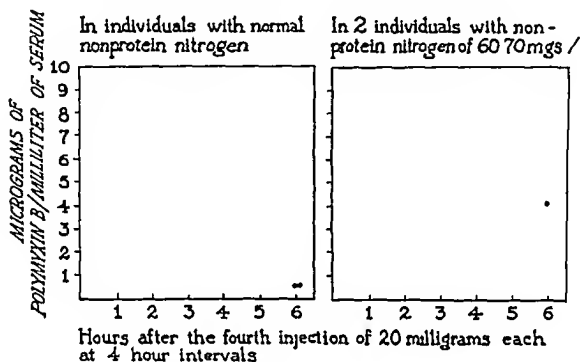


Fig 2

*4 Serum Levels of Polymyxin B in Patients With Urinary Tract Infection*—For intramuscular administration, solutions of Polymyxin B were prepared by adding 2 ml of saline and 1 ml of procaine, 1 per cent, to the dry amorphous material equivalent to 40 mg of pure drug. Without the local anesthetic the injection was painful and tenderness persisted for some hours. Ordinarily 20 mg of drug were injected in a volume of 1.5 ml for each dose. In a few instances 10 mg were administered for from one to four doses. Injections were given every four to eight hours.

Early observations indicated accumulation of the drug, administered with procaine every four to eight hours. Consequently levels in serum were estimated usually after multiple doses. Fig 2 shows the levels in four patients in whom there was no evidence of nitrogen retention from one to six hours after the fourth dose of 20 mg Polymyxin B administered every four hours. In individuals with impaired renal function and retention of nitrogenous waste products there was an increased accumulation of the drug (Fig 2). In such patients serum levels of 2 to 5 µg per milliliter usually could be maintained by the injection of 20 mg Polymyxin B every eight hours, and measurable levels of the drug could be detected eighteen to twenty-four hours after the last of eight to twelve injections.

In two patients, four hours after a single injection of 40 mg, the serum concentration of Polymyxin B was 13 to 20  $\mu\text{g}$  per milliliter. After four such injections (with procaine), the serum levels were, respectively, 30 and 20  $\mu\text{g}$  at two hours, 5 and 8  $\mu\text{g}$  at seven hours, and 3 and 6  $\mu\text{g}$  at twelve hours after the fourth injection. There were toxic symptoms in both patients during this period.

Measurement of the drug in the urine was not satisfactory. Filtration through Sertz pads removed a considerable portion of Polymyxin B, and in boiled urine the drug levels were very irregular.

*5 Therapeutic Results in Patients With Urinary Tract Infection*—Ten patients with acute or subacute exacerbations of chronic urinary tract infections were treated. They had little or no obstructive uropathy, and all had failed to respond to therapy with sulfonamides and streptomycin. The infecting agents were uniformly resistant to streptomycin *in vitro*. Before, during, and after treatment, a complete blood count, urinalysis, phenolsul fophthalein test, and determination of nonprotein nitrogen in the blood were obtained for every patient. The subjects were hospitalized and closely observed clinically, and quantitative bacteriologic studies were carried out on the catheterized urine every two days during therapy and at frequent intervals thereafter.\* Most patients were afebrile.

Polymyxin was injected intramuscularly in amounts of 20 mg every four to eight hours for three or four days. By frequent determinations, an effort was made to keep serum levels between 2 and 10  $\mu\text{g}$  per milliliter. Some of the data obtained in these patients are shown in Table III. When the infecting organism was inhibited *in vitro* by 0.4  $\mu\text{g}$  per milliliter, the short course of therapy with Polymyxin B led to negative urine cultures for one to three weeks. Bacteriologic relapses occurred, usually within one month, even when the patients remained symptomatically well. A detailed example of this temporary response to therapy is shown in Table IV.

*6 Toxicity*—After intramuscular injection of Polymyxin B (13 mg per milliliter), most subjects experienced local pain with persistent tenderness severe enough to warrant the addition of a local anesthetic for routine administration of the drug. With procaine the injections were well tolerated.

In spite of careful search, no evidence of renal damage could be detected with the short course (three to four days) of Polymyxin B used in this study. There was no hematuria or proteinuria and there were no changes in the sediment or nitrogen retention during or after therapy. The proteinuria of patients with chronic pyelonephritis was not aggravated by the drug. This is in contrast with the experience of others<sup>16</sup> who found proteinuria and hematuria at least transiently when the drug was given for more than seven days.

One of the patients developed a drug fever on the third day of treatment. It promptly subsided when the injections were stopped, but recurred when

\*We wish to thank Dr. Frank Hinman, Dr. Frank Hinman Jr., Dr. Frederick S. Howard, Dr. John W. Schulte and others, and the members of the resident staff of the University of California Hospital and Franklin Hospital for permission to study their patients.

TABLE III TREATMENT OF PATIENTS WITH URINARY TRACT INFECTION WITH POLYMYXIN B BACTERIOLOGIC RESULTS

PATIENT	POLYMYXIN B DOSE			INFECTING ORGANISMS	SENSITIVITY ( $\mu$ O/MIL)	URINE CULTURES (ESTIMATED NUMBER OF BACTERIA/MIL)					CLINICAL RESPONSE
	TOTAL MG	MG/KG /DAY	DAYS TREATED			BEFORE R	AT END OF R	AFTER TREATMENT			
								1 WK	2 WK	3 WK	
JT	480	1.7	4	A aerogenes	0.2	500 000	0	0	100	10 000	Good
EM	400	1.8	4	Esch coli	0.1	80 000	0	0	0	20 000	Good
MAS				Proteus sp	0.8	1 000	10	10	50	500	
	360	2.9	3	P <sub>a</sub> aeruginosa	0.4	30 000	0	0	-	0	Good
	360	1.9	3	Esch coli	0.1	2 mill	0	0	-	400 000	Good
	470	2.2	4	A aerogenes	0.4	100 000	0	2 000	-	10 000	Fair
RP	360	3.1	3	Esch coli	0.1	1 mill	0	0	5 000	50 000	Fair
MIS	480	3.0	4	Esch coli	0.2	200 mill	0	0	-	-	Fair
				Str faecalis	10.0	50 000	1 mill	100 000	-	-	
MIV	300	2.3	3	A aerogenes	0.4	50 000	20	100 000	10	5 000	Poor
				P <sub>a</sub> aeruginosa	2.0	50	10 000	1 000	1 mill	10 000	
IF	420	2.1	4	S alkalescens	2.0	10 mill	200 000	50 mill	-	-	Died
	480	3.6	3	P <sub>a</sub> aeruginosa	1.0	500 000	10 000	1 mill	-	-	Poor

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Ataxia	0	-	-	+	-	-	-	-	-
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Bacteria/high dry field	++++	+++	++	+	Rare	Rare	0	0	+++
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## DISCUSSION

The known antibiotic substances derived from *B. polymyxa* share a specific and powerful antibacterial action against gram negative bacteria. These peptides are bactericidal rather than bacteriostatic in vitro for susceptible bacteria. Resistant bacterial variants emerge infrequently. All these characteristics would make this group of compounds desirable in the treatment of infections with gram negative organisms were it not for significant toxic side effects.

Among organisms encountered in urinary tract infections susceptibility to Polymyxin B was uniformly high among strains of *Esch. coli*, and only slightly less with *A. aerogenes*. Many strains of *Pseudomonas* and *Proteus* on the other hand were fully resistant. This parallels the experience with other antibiotics especially streptomycin and to a lesser extent aureomycin.<sup>1</sup> In some strains, resistance to Polymyxin B was induced in vitro with ease. The change did not appear to be related to the original sensitivity to the drug in the four strains tested. Studies based on large inocula suggested that the proportion of resistant organisms in a given bacterial population was small. Resistant variants did not develop in patients during a short course of treatment with Polymyxin B.

The natural history of chronic infection of the urinary tract does not support the idea that a short course of any antibacterial agent could "cure" the disease. Bacteria within the inflamed renal parenchyma and the proliferating fibrous tissue are shielded effectively from the immediate action of drugs. Prolonged therapy at best would be necessary to eradicate the infective organisms. In the present study no such attempt was made. Treatment with 300 to 500 mg of Aerosporin administered in three to four days resulted in a striking reduction of the bacterial content of the urine for one to three weeks in six of ten patients. Symptomatic improvement was satisfactory and usually lasted longer than the bacteriologic remission. Failure to respond was associated with relative resistance of the infecting organisms or with multiple infection. Within its scope the therapy may be said to have been successful. Whether prolonged treatment might yield more encouraging results cannot be predicted.

Trials of prolonged therapy were made inadvisable by toxic effects from the Polymyxin B used. The most dramatic were the neurological symptoms and signs which appeared in a majority of patients. Although these disturbances disappeared promptly after therapy was discontinued and did not leave any residual changes patients were uncomfortable and apprehensive; some refused further treatment. No renal toxicity or untoward effects on the hematopoietic system were noted with the short course of therapy used in this study.

The described side effects of Polymyxin B preclude the clinical use of the preparations presently available. The antibacterial features of the drug are so attractive however that it must be hoped that the toxic properties can be eliminated in the future and the agent made available for further clinical investigation.

## SUMMARY

1 Aerosporin brand Polymyxin B is a stable peptide derived from *B polymyxa* with marked antibacterial action against many gram-negative organisms. Among bacteria commonly isolated from urinary tract infections, *Esch coli* and *A aerogenes* were inhibited in vitro by less than 1  $\mu$ g per milliliter, while many strains of *Proteus* and *Pseudomonas* were more resistant.

2 Resistance could be induced in susceptible bacterial strains only with difficulty. The action of the antibiotic in vitro appeared to be bactericidal rather than bacteriostatic.

3 Multiple intramuscular injections of the drug resulted in cumulative serum levels. The serum levels were greatly increased in the presence of elevated nonprotein nitrogen of the blood.

4 In patients with chronic urinary tract infection, Polymyxin B administered for three to four days eliminated susceptible bacteria from the urine and controlled symptoms. The short course of treatment, however, was followed by a bacteriologic relapse within one to three weeks in all cases.

5 Prolonged therapy with the drug was not feasible because of toxic symptoms affecting particularly the nervous system. In its present form, Polymyxin B is therefore not suitable for general clinical application.

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## THE COAGULATION DEFECT IN THROMBOCYTOPENIC PURPURA

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**T**ILL normal coagulation time observed in thrombocytopenic purpura has always puzzled students of coagulation and has led many to conclude that coagulation is not disturbed in this disease. Recently evidence has accumulated to show that the coagulation time may not always be a reliable measure of true coagulability.<sup>1</sup> In fact, it has long been recognized that in hypoprothrombinemia the coagulation time is much too insensitive to serve as a guide for estimating the severity of prothrombin deficiency. As a result of the development of the prothrombin time determination rapid progress, both clinical and theoretical, ensued in the group of diseases in which a deficiency of prothrombin occurs. The lack of a test comparable to the prothrombin time procedure to measure quantitatively the factors responsible for the activation of prothrombin has hampered and retarded progress in the study of such diseases as hemophilia and thrombocytopenic purpura. With the development of the prothrombin consumption time (serum prothrombin time) a new approach is offered for the study of the hemorrhagic diseases due to coagulation defects which are not in the prothrombin complex, i.e., diseases in which the prothrombin time is normal. With this test Quick<sup>2</sup> demonstrated that the prothrombin consumption was markedly delayed and incomplete in hemophilia and likewise in plasma from which most of the platelets were removed. These findings were subsequently verified by Soulier.<sup>3</sup> Earlier Brinkhous<sup>4</sup> had noted the incomplete conversion of prothrombin in clotted hemophilic blood which he attributed to a slow conversion rate of the prothrombin.

Clot retraction which is characteristically absent in severe thrombocytopenic purpura should no doubt be considered as a coagulation defect although it is difficult to find in the literature specific statements to support such a view. While it is recognized that clot retraction is dependent upon intact platelets a quantitative correlation between the number of platelets and the speed and degree of clot retraction was difficult to make until the introduction of silicone (Dri film) which made it possible to secure and keep native plasma with a minimum amount of platelet lysis. Whole blood is far less satisfactory for studying clot retraction since variations in cell volume markedly influence the

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TABLE I A CORRELATION OF THE PLATELET COUNT WITH THE BEGINNING OF CLOT RETRACTION AND THE PROTHROMBIN CONSUMPTION TIME IN NORMAL SUBJECTS

SUBJECT	PLATELET COUNT (THOUSANDS)		HEMATOCRIT READING (PLASMA VOLUME)		BEGINNING OF CLOT RE TRACTION (MIN)	PROTHROMBIN CON SUMPTION TIME (AFTER 60 MIN)	
	WHOLE BLOOD	NATIVE PLASMA	RELAT- IVE* (%)	TRUE (%)		BLOOD (SEC)	PLASMA (SEC)
1	204	486	8	48	9½	34	48
2	154	535	22	50	9½	17	23
3	282	46	42	44	7	16	42
4	354	800	22	47	6½	19	23
5	252	187	26	49	6	18	18
6	201	352	28	51	8½	15	23
7	177	345	38	46	6½	19	29
8	207	449	24	49	8½	21	27
9	255	583	38	48	10	36	26
10	241	498	26	47	8	23	34
11	209	406	28	45	6½	23	25
12	284	583	16	43	8½	27	24
13	197	576	29	48	11	23	23
14	259	662	24	48	9	17	27
15	214	508	34	47	7½	23	28
16	207	604	38	51	7½	21	20
17	300	770	36	45	7	20	19
18	205	549	30	47	6	32	18
19	422	611	36	51	9	21	31
20	322	083	32	49	8	17	18
Average	250	531	29	47	8	22	26

\* Ratio of plasma volume obtained by centrifuging at 300 r p m for ten minutes to total blood volume.

When the platelet count is normal, clot retraction of native plasma becomes detectable in about eight minutes. The range for the twenty subjects studied varied from six to eleven minutes. In confirmation of previous studies,<sup>6</sup> it was observed that when the platelet count of plasma is below approximately 150,000 the beginning of clot retraction becomes progressively delayed and finally retraction fails to occur altogether. Slight retraction still manifests itself when the count is over 20,000. Under ideal experimental conditions and with control of the various clotting factors the number of platelets quantitatively determines the speed with which clot retraction begins and also the size of the final clot.

The prothrombin consumption time likewise, depends on an adequate number of platelets. It has been postulated that platelets supply an enzyme which is essential for the conversion of thromboplastinogen to active thromboplastin. On removing platelets from plasma by centrifugation the prothrombin consumption is markedly delayed due to the inadequate conversion of thromboplastinogen. With an excess of platelets, sufficient platelet enzyme becomes available to permit a rapid consumption of prothrombin. Inspection of Table I shows that the prothrombin consumption time of serum, whether obtained from plasma or whole blood sixty minutes after coagulation is in most instances between 17 and 30 seconds. If these values are calculated in terms of prothrombin activity by means of Quick's prothrombin curve, one finds that from 60 to 80 per cent of the activity disappears in one hour.

A poor consumption of prothrombin is due generally either to a lack of platelets or to a deficiency of thromboplastinogen. In hemophilia the platelets are normal, since it has been shown that if platelets from hemophilic blood are added to normal deplateletized plasma, normal coagulation will occur.<sup>7</sup> It is most likely therefore that the basic defect in hemophilia is inadequate thromboplastinogen content in the blood.

To determine whether defects in coagulation other than lack of platelets occur in thrombocytopenic purpura, one must consider the various factors that enter into the clotting mechanism. Since the prothrombin time is usually entirely normal, it is evident that the prothrombin complex and fibrinogen are not involved and are normal. This is clearly illustrated by the following findings made on the blood and native plasma of a patient with severe thrombocytopenic purpura:

PATIENT R. P., 11 YEAR OLD GIRL

Coagulation time (Lee-White)	6 min
Prothrombin time	11½ sec
Prothrombin consumption time (whole blood)	10½ sec (after 1 hr)
Bleeding time	>30 min
Clot retraction (whole blood)	None (after 24 hr)
Platelet count	5,000 per cu mm

STUDY OF NATIVE PLASMA (OBTAINED BY SILICONE TECHNIQUE)

Coagulation began in	5 min
Coagulation completed in	12 min
Retraction	None in 2 hr
Prothrombin consumption time	10½ sec (after 2 hr)

From these results it can be seen that only a trace of prothrombin was consumed in the clotted blood after two hours. That this is due to lack of active thromboplastin is clearly shown by the effect of adding a small amount of rabbit brain extract to the serum (Table II). An immediate increase in the prothrombin consumption occurred, and when the amount of added thromboplastin was increased, the prothrombin consumption was also proportionately increased. Since the prothrombin consumption time tends to remain fairly constant for any concentration of thromboplastin for a period of one hour or more after its addition, it appears very likely that the reaction with prothrombin is stoichiometric rather than enzymatic. The lack of active thromboplastin is not due to a deficiency of thromboplastin as occurs in hemophilia, but to an inadequate amount of the activator which is furnished by the platelets.

TABLE II THE EFFECT OF ADDING THROMBOPLASTIN TO SERUM OF A PATIENT WITH THROMBOCYTOPENIC PURPURA ON PROTHROMBIN CONSUMPTION TIME

	PROTHROMBIN CONSUMPTION TIME			
	TIME IN MIN			
	0	15	30	60
Thromboplastin added to 1 cc of serum		21		
01 cc	10½	41	21	22
02 cc	10½		—	44

The correlation of prothrombin consumption time with the beginning of clot retraction time can be applied to study the course of the thrombocytopenic purpura, as illustrated by two cases, the laboratory findings of which are summarized in Tables III and IV

TABLE III THE COURSE OF THROMBOCYTOPENIC PURPURA AS MANIFESTED BY THE PLATELET COUNT, BLEEDING TIME, BEGINNING OF CLOT RETRACTION IN NATIVE PLASMA, AND PROTHROMBIN CONSUMPTION TIME

(Patient A boy, age 0, Diagnosis Secondary thrombocytopenic purpura)

DATE	PLATELET COUNT	BLEEDING TIME (MIN)	BEGINNING OF CLOT RETRACTION (PLASMA) (MIN)	PROTHROMBIN CONSUMPTION TIME (WHOLE BLOOD) (SEC)	CLINICAL CONDITION
June 4	20,000		27	13	Marked bruising
June 8	25,000	10	29	11	Marked bruising
June 12	49,000	5½	18	13	Marked bruising
June 17	13,000	10	15	13	Marked bruising
June 24*	15,000	1½		11	Marked bruising
July 1	29,000	9½	14	14	Marked bruising
July 14	98,000	2½	11	20	No bruising

\*Folic acid 20 mg daily

TABLE IV THE EFFECT OF SPLENECTOMY ON THE PLATELET COUNT AND PROTHROMBIN CONSUMPTION TIME IN A PATIENT WITH THROMBOCYTOPENIC PURPURA

(Patient A man age 32, Diagnosis Idiopathic thrombocytopenic purpura)

DATE (1948)	PLATELET COUNT OF WHOLE BLOOD (THOUSANDS)	CLOT RE TRACTION OF WHOLE BLOOD	PROTHROMBIN CONSUMPTION TIME (SEC)	REMARKS
April 1	80,000	None	10	
June 24	14,000	None	10	
June 25	39,000	Slight	12	Immediately after ligation of splenic vessel
	22,000		13	After 30 min.
	20,000		14	After 60 min.
June 26	98,000	12½ min *	32	
June 28	342,000		50	

\*Native plasma

The first subject, a 6 year old boy, developed purpura two weeks following an attack of vomiting and diarrhea. He had no hematuria, melena or other type of bleeding except marked bruising. He had a consistently low platelet count over a period of six weeks. During this period the bleeding time was prolonged, the beginning of clot retraction delayed, and the prothrombin consumption time consistently shorter than normal. The clinical condition as judged by marked bruising remained unchanged. Two weeks following the daily administration of 20 mg folic acid, the bruising tendency disappeared, and an examination of the blood showed a striking increase in the platelet count and a return to normal of the bleeding time, clot retraction, and prothrombin consumption time. Whether the cure can be attributed to folic acid or whether recovery was spontaneous remains problematic, although favorable results from folic acid have been noted also in a few other cases of thrombocytopenia.

The second subject,\* a young man 32 years of age with idiopathic thrombocytopenic purpura, presented the opportunity to study the effect of splenectomy. It is obvious from the results recorded in Table IV that the favorable response to the removal of the spleen is shown both by the rise in the platelet count and by the increase in prothrombin consumption. It appears that the latter was even a more sensitive indicator than the platelet count, but that may perhaps be explained by the difficulty of avoiding errors in counting platelets especially when the collection of blood is beset with technical difficulties, such as are encountered in the operating room with the patient under anesthesia.

In both patients the prothrombin consumption time corresponded to the platelet count, and clinical improvement was promptly and accurately indicated by a rise both in the platelet count and in the prothrombin consumption.

The prothrombin consumption time test is at present the only method available to show a coagulation defect in thrombocytopenic purpura. When performed according to the directions as outlined in this paper, it is a fairly quantitative measure of platelet activity unless there is a deficiency of thromboplastinogen as in hemophilia. The test therefore becomes a useful addition to the list of laboratory procedures available for studying purpura. It will definitely complement the platelet count. From the studies made thus far, it can be stated that when the prothrombin consumption time is normal, no marked thrombocytopenia exists.

The new method for studying clot retraction by timing the beginning of the process in native plasma is a far more sensitive test than the clot retraction of whole blood. The latter method is essentially qualitative and therefore is only a rough index of the platelet count. Unfortunately the collection of native plasma is too exacting a procedure to warrant its general use in the clinical laboratory, but for investigative purposes, plasma is better suited to study clot retraction than is whole blood since the reaction can be directly watched and no correction for cell volume is required.

A close correlation exists between the platelet count, clot retraction, and the prothrombin consumption time since the latter two are directly dependent upon the former, assuming of course, that the activity per platelet is constant. The prolonged bleeding time, the easy bruising, the oozing of blood from mucous membranes, and the petechiae which are characteristic findings in thrombocytopenic purpura are in all probability not caused by the platelet deficiency. It is not unusual to find in such conditions as acute lymphatic leukemia a very low platelet count and yet no purpura or bleeding. It seems fairly certain that a toxic agent distinct from the platelets is responsible for the vascular dysfunction that causes purpura and that when this factor is superimposed on the coagulation defect due to lack of platelets, the hemorrhagic tendency is markedly accentuated.

#### SUMMARY

A correlation between the platelet count, the speed of clot retraction, and prothrombin consumption of normal human native plasma (obtained with the

\*The medical staff at Veterans Hospital Wood Wis kindly permitted us to study this patient.



and of silicone coated glassware) is presented. Incidental to this study, it was observed that the platelet count in plasma obtained by slow centrifugation is about twice as high as in whole blood.

In thrombocytopenic purpura a delayed clot retraction and a poor prothrombin consumption closely parallels the low platelet count. A clinical case is presented to illustrate the simultaneous and prompt return to normal of the prothrombin consumption time and the speed of clot retraction as the platelet count increased to 100 000 and the clinical condition improved. Another case is selected to show the expeditious response of the platelet count, prothrombin consumption, and clot retraction to splenectomy in idiopathic thrombocytopenic purpura (Weilhof's disease).

The prothrombin consumption test supplies the first strong evidence that a coagulation defect is present in thrombocytopenic purpura. The test offers a new measure of platelet activity, which is postulated to be that of activating plasma thromboplastinogen. Clot retraction likewise is a measure of intact platelet activity. When the test is carried out on native plasma, it has quantitative significance since the beginning and completion can be accurately determined by direct visual observation, and no correction is required for cell volume.

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Patient 6 had an acquired hemolytic anemia four years previously which responded well to splenectomy. Although the anemia disappeared and there were no signs of increased blood destruction, spherocytosis continued and the developing test was positive. The patient died in anemia consequent to malignant hypertension.

Patient 7 had a typical symptomatic hemolytic anemia occurring in a case of reticulum cell sarcoma. Although slight spherocytosis was present in the film, the hypotonic saline fragility was within normal limits. Coating antibodies in cases of symptomatic hemolytic anemia have been observed by Boorman and associates<sup>10</sup> and Wagley and co-workers.<sup>17</sup>

Patient 8 showed a strongly positive Coombs test before splenectomy in the presence of a very severe anemia and 57 per cent reticulocytes. Two months after splenectomy, spherocytosis and the increased saline fragility disappeared and the hemoglobin and red cell count returned to normal values. The developing test also became negative. This case is an example of a complete cure of this disease which sometimes follows splenectomy.

**2 Hereditary Spherocytic Anemia**—Seven patients were examined (Table II). All of them had the typical features of spherocytosis and increased saline fragility. All but one had a negative developing test. Splenectomy was performed on Patient 3 only.

TABLE II RESULTS OF DEVELOPING TEST IN CONGENITAL HEMOLYTIC ANEMIA

PATIENT	AGE (YR)	SEX	HB (GM)	RBC (MILL)	RETIC (%)	DEVELOPING TEST	REMARKS
1	28	F	10.6	3.2	10.6	Negative	Diagnosis based on developing test only as family not available for examination
2	23	F	10.6	3.2	13.0	Negative	Son of Patient 2. Developing test still positive 12 days after splenectomy. Six weeks following operation test turned negative.
3	4	M	3.9	1.8	10.4	Positive	
4	49	F	11.7	3.6	6.6	Negative	Sister of Patient 4
5	45	F	11.7	3.5	8.6	Negative	
6	55	F	11.7	3.8	4.4	Negative	
7	21	M	14.3	4.6	0.2	Negative	Son of Patient 6

Two cases in this group are of particular interest. Patient 1 had a negative Coombs test on two occasions. It was not possible to examine the family. The classification of this case is therefore arbitrary and based only on the negative developing test.

Patient 3, the son of Patient 2, presented the most interesting problem—a positive developing test in a well established case of hereditary hemolytic anemia. This 4-year-old white boy entered the Sarah Morris Hospital because of accidental trauma. His mother (Patient 2) had always been anemic and had been advised to have a splenectomy several years ago. The child had been pale and icteric since the age of 4 months. Splenectomy, although repeatedly advised, had always been refused. Transfusions also had never been given.

Physical examination revealed a mildly jaundiced child with a large hematoma about the forehead. There was a loud systolic murmur at the apex. The liver was palpable 4 cm below the costal margin in the midclavicular line, and the spleen reached almost down to the iliac crest and had a definite palpable notch. Skull and chest films were within normal limits.

**Laboratory Data** Hematologic findings: Hb, 3.9 Gm (26 per cent) per 100 ml, RBC, 1.8 mill per cubic millimeter; WBC, 4,700; reticulocytes, 10.4 per cent; hematocrit, 12; CI, 0.72; MCV, 70 cu mm; platelets, 164,800. The blood film showed conspicuous

hypochromia and microcytosis as well as definite spherocytosis. The coexistence of both poorly stained hypochromic corpuscles and fully hemoglobinized spherocytes was very striking. The differential count was within normal limits. Saline fragility started at 0.8 per cent. Bleeding, clotting and prothrombin times were normal. Blood group formula: A MN, Rh<sub>1</sub> Rh<sub>2</sub> ( $\frac{CDE}{c}$ )\*. Bone marrow marked erythroid hyperplasia of normoblastic variety.

**Chemistry.** Serum bilirubin 3 mg per cent, cholesterol, 78 mg per cent (60 per cent esters), thymol turbidity 2.4 units, cephalin and thymol flocculation tests, negative, total protein, 7.2 Gm, true albumin 4.4 Gm, true globulin, 2.8 grams. The Kahn test was negative and the urine did not contain bilirubin.

The patient was splenectomized after the hemoglobin and cell count were brought to normal levels by means of several blood transfusions. He made an uneventful recovery. His blood findings were within normal limits seven weeks after splenectomy, and he is continuing to do well.

It should be emphasized that it is difficult to decide whether this patient was in crisis when he entered the hospital. His mother stated that his general condition did not differ from the usual and that she brought him to the hospital because of the trauma. Although blood examination revealed a very severe anemia, a slight leucopenia and thrombocytopenia, there was a definite elevation of reticulocytes and the marrow did not show any evidence of maturation arrest as described by Owen.<sup>19</sup> The hypochromia probably was caused by poor nutritional intake in this growing chronically ill patient.

The developing test was strongly positive on admission, and the same result was obtained five days later after several transfusions were given. On the twelfth day following splenectomy the test still was strongly positive but became negative five weeks later. The disappearance of the positive developing test after splenectomy should be stressed. Some of our own and previously published cases of acquired hemolytic anemia<sup>10, 13, 18</sup> showed persistence of a positive developing test for long periods after operation.

**3 Sickle Cell Anemia in Crisis.**—Two patients with sickle cell anemia in crisis showed a negative developing test thus demonstrating that no coated cells are present during these episodes. This finding points against an immunologic extracorporeal mechanism operating during the crises of this disease.

**4 Miscellaneous.**—One syphilitic patient with a severe nonspherocytic hemolytic anemia and hepatitis probably caused by arsphenamine, showed a negative Coombs' test. Negative results also were obtained in a case of chronic myelogenous leukemia with strong autoagglutination and in another patient with spherocytic hemolytic anemia caused by a new sulfa compound (Sulphonation).

#### 5 Results of Other Investigators—

(a) *Acquired Hemolytic Anemia (Idiopathic and Symptomatic).* The results of the developing test as obtained by other investigators are tabulated in Table III. In all of the twenty-four previously reported cases a positive developing test was regularly found. No negative results were encountered. Splenectomy did not lead to disappearance of the coated spherocytes although the hemolytic process had stopped. Boorman and co-workers<sup>10</sup> found a negative Coombs test

We are indebted to Dr. S. O. Ievins and Dr. A. Wolf of the Serum Center, Michael Reese Hospital, for establishing the exact Rh-Hr formula of this patient.

in five atypical nonspherocytic cases of acquired hemolytic anemia. These cases are omitted from this discussion as they do not exhibit the diagnostic criteria of a spherocytic acholuric jaundice. Loutit and Mollison<sup>18</sup> added two more cases to those already reported by Boorman and associates. One of these patients had a positive developing test, although it is stated that at the time of testing there was no spherocytosis and a normal saline fragility. This patient appears to have had a typical spherocytic hemolytic anemia, and she later died of this disease.

TABLE III RESULTS OF OTHER INVESTIGATORS

INVESTIGATORS	NUMBER OF CASES OF CONGENITAL HEMOLYTIC ANEMIA WITH NEGATIVE DEVELOPING TEST			NUMBER OF CASES OF ACQUIRED HEMOLYTIC ANEMIA WITH POSITIVE DEVELOPING TEST			REMARKS
	TOTAL	BEFORE SPLENECTOMY	AFTER SPLENECTOMY	TOTAL	BEFORE SPLENECTOMY	AFTER SPLENECTOMY	
Boorman and co workers <sup>10</sup>	17	8	9	5	2	3	One case of acquired hemolytic anemia of symptomatic variety. Persistence of positive developing test in one patient up to 2 yr after splenectomy.
Loutit and Mollison <sup>18</sup>	0	0	0	2	2	0	Strongly positive developing test in one case in absence of spherocytes and increased fragility. Patient had spherocytosis prior to test and later died of acholuric jaundice.
Evans and co workers <sup>14</sup>	0	0	0	2	2	0	One patient with sickle cell anemia and two patients with Cooley's anemia also showed negative developing test.
Sturgeon <sup>15</sup>	3	3	0	3	1	2	
Wagley and co workers <sup>17</sup>	3	3	0	12	Not specified	Not specified	At least two of the acquired cases are of the symptomatic type.

(b) *Hereditary Hemolytic Anemia* A negative developing test was found in all of the twenty-three cases reported so far (Table III). Our case showing a positive Coombs test appears to be the first observation of such an occurrence in the hereditary disease. However, Dameshek and Bloom,<sup>20</sup> without actually performing a developing test, have demonstrated incomplete antibodies in the plasma of two patients with hereditary hemolytic anemia in crisis using albumin as a diluent. Since in the presence of such antibodies the developing test always has been found positive, these two cases may be quoted as corroborative evidence.

#### DISCUSSION

The interpretation of a positive developing test in acquired and in some instances of hereditary spherocytic jaundice requires a critical understanding of the pertinent facts concerning the pathogenesis of these syndromes. Because

of the controversial character of the currently favored theories it was deemed necessary to correlate and to consider these problems in greater detail

#### *A Theoretical Considerations—*

*Immunologic Nature of the Developing Test* Recent work has clearly established the existence of at least three different types of antibodies to the Rh factors. These are (1) the saline (complete, first order) antibody, (2) the albumin (incomplete, saline blocking,<sup>21, 22</sup> second order) antibody, and (3) the "true" blocking<sup>23</sup> (cryptagglutinoid<sup>23</sup> third order) antibody. The second order antibody blocks the reactivity of the saline antibody and can be removed from the red cells by extensive washing. The "true" blocking antibody blocks the reaction of the albumin agglutinin and cannot be washed off.<sup>23</sup>

Recently Mohn and Witebsky<sup>23, 24</sup> have shown that the last two mentioned varieties of immune bodies cause "coating" of the red cells. Consequently a positive developing test is indicative of the adsorption of either the second or third order antibodies onto the surface of the red cells. Failure to obtain agglutination in albumin in the presence of a positive developing test clearly establishes the existence of this "true" (third order) blocking antibody.

A positive developing test in spherocytic hemolytic anemia has been repeatedly demonstrated without any evidence of antibodies directed against the Rh Hr (CDE) system. It may be assumed that the agglutinins found in the spherocytic hemolytic anemias follow a similar immunologic pattern. Nothing is known, however, about antigenic or other mechanisms stimulating the production of these immune bodies.

The demonstration of a positive developing test in our case of hereditary hemolytic jaundice and the corroborative results of Dameshek and Bloom<sup>6</sup> prove the presence of an immunologic mechanism in these cases. Anti Rh Hr immune bodies are unlikely to be responsible for the positive developing test because our patient was Rh positive and splenectomy resulted in the disappearance of the positive Coombs test.

*Nature and Origin of Spherocytosis* Spherocytes are red cells with increased thickness, decreased mean cell diameter and normal volume. These physical alterations account for the globular shape of these cells as contrasted to the biconcavity of the normal erythrocytes. Spherocytosis may be found in a great variety of conditions. Experimentally it may be produced *in vivo* by burns<sup>25</sup> and by the injection of certain hemolytic agents (e.g. antineoplastic cell sera<sup>26</sup> phenylhydrazine). *In vitro* it may be brought about by exposing red cells to certain chemicals (e.g. lysolecithin saponin) or to lowered osmotic tension or even by firmly pressing a cover slip over a fresh preparation of blood.<sup>27</sup> Clinically, spherocytosis is always found in the hereditary type and in many cases of the acquired type of hemolytic anemias of various etiologies (immunologic, physical and chemical).

These observations clearly demonstrate that a multitude of mechanisms may be responsible for this change in the shape of the red cell. The common result of all these conditions is an alteration in the structure of the erythrocytes, leading to a disturbed equilibrium of the forces which keep the normal corpuscles in a biconcave shape. The nature of these forces is but poorly understood at the present time.<sup>28</sup>

Since the number of biologically significant alterations in shape which a red cell may assume appears to be limited, it is not permissible to deduce that a given morphologic anomaly is always produced by the same factors. Furthermore, the functional behavior of red cells having the same abnormal shape may vary in many ways as determined by the original etiologic mechanisms. Functional differences between spherocytes are clearly shown by the reaction of these cells to the developing and the lysocerithin fragility tests. Recent survival time studies with sickle cell anemia and sickle cell trait cells also point to qualitative variations hiding behind the common feature of the sickling tendency.<sup>27-28</sup>

The recognition that several types of spherocytes exist must be taken into consideration by any theory as to the site of origin of this anomaly in shape which occurs in various clinical conditions. The spherocytes found in the circulation of severely burnt patients quite obviously originate in the damaged areas. It appears more than likely that the qualitatively different spherocytes found in the hereditary and acquired (immunologic) types of hemolytic anemia are also of different origin. This point of view may be helpful in evaluating the current hypotheses on the origin of spherocytosis.

According to Naegeli<sup>29</sup> the shape of any erythrocyte is primarily regulated in the marrow. In hereditary hemolytic jaundice many red cell precursors are, therefore, predestined to become spherocytes. According to this concept spherocytosis is a red cell anomaly under genetic control. The failure of spherocytosis to disappear following splenectomy in congenital acholic jaundice is often cited to support this theory. It should be emphasized, however, that many cases of acquired hemolytic anemia also show persistent spherocytosis after splenectomy and that a positive developing test may remain demonstrable for many years.

Dameshek<sup>30</sup> objects to Naegeli's hypothesis on the ground that spherocytosis is never observed in the nucleated and reticulated red cells of the marrow. He postulates the action of an intravascular antibody on normal cells, thus rendering them spherocytic. According to this unitarian hypothesis, any spherocytosis—congenital or acquired—is caused by extracorporeal mechanisms. The fact that normal erythrocytes transfused into a patient with hereditary hemolytic anemia have a normal life span whereas the patient's own cells are prematurely destroyed<sup>8-30</sup> is explained by the assumption that the postulated immune bodies are "autospecific"—attacking only the patient's own erythrocytes. Such an interpretation necessarily implies that the production of these hypothetical "auto-specific antibodies" is also controlled by an hereditary mechanism. It should be pointed out that the failure of nucleated and reticulated erythrocytes to exhibit spherical shape cannot be used to disprove their primary origin in the marrow. It is quite possible that the presence of nuclear material so alters the cytoskeleton that spheroidicity cannot be achieved. The fact that in nonhemolytic elliptocytosis the nucleated corpuscles also fail to show elliptic shape<sup>31</sup> may be quoted as another example of a genetic blood disorder not displaying the characteristic shape anomaly in the nucleated precursors.

In regard to the "autospecific" immune bodies it should be stated that the developing test is an unspecific test for globulin adsorbed to the surface of the erythrocytes. Since spherocytes found in the hereditary disease are usually

"uncoated" it follows that the hypothetical autoagglutinins or hemolysins are either not globulins or that the test is not sensitive enough to detect them. Even if such factors should be discovered it is more than likely that they would be found to differ qualitatively from the globulin immune bodies.

Ham and Castle<sup>1</sup> consider stasis to be an important factor producing spherocytosis. These investigators have shown that in vitro incubation of red cells and also stagnation of erythrocytes in the spleen result in increasing spheroidicity with increasing mechanical and osmotic fragility. Erythrostasis is supposed to cause an accumulation of metabolites with resulting swelling of the cells. This hypothesis does not account for the absence of spherocytosis in many splenomegalias (e.g., polycythemia) where severe congestion and stasis may be found.

Most of these hypotheses are *unitarian* in character, either disregarding the demonstrable qualitative differences of the spherocytes or requiring special assumptions for a satisfactory explanation of the known facts. Although these theories have been extremely useful in stimulating valuable experiments which uncovered new evidence we believe that at present the hypothesis of the *multiple origin of spherocytosis* is more adequate.

From this standpoint it seems quite probable that in the hereditary type of hemolytic anemia uncoated spherocytes develop in the marrow under genetic control whereas in the acquired variety spherocytes are formed after contact with coating antibodies originating in the spleen<sup>1</sup> or possibly other organs. Spherocytosis caused by physical and chemical factors results from the exposure of the erythrocytes to these agents either intravascularly or within the damaged tissue.

Fig. 1 represents a diagram summarizing the pertinent points of this hypothesis.

The exceptional presence of coated spherocytes in a proved case of hereditary hemolytic anemia is therefore interpreted as being caused by the simultaneous existence of two pathogenic mechanisms. After splenectomy the production of coating antibodies ceased and the developing test became negative. Spherocytes of the uncoated variety continued to be present.

*The Role of the Spleen in the Spherocytic Hemolytic Syndromes.* In hereditary spherocytic anemia the pathologically increased destruction of red cells disappears following splenectomy. It is usually assumed that the spleen participates physiologically in erythrocyte disintegration and that in the spherocytic anemia a hyperfunction (hemolytic hypersplenism) exists. Since the mode of red cell elimination under normal conditions is still a matter of debate various mechanisms like erythrophagocytosis,<sup>33</sup> erythrostasis,<sup>32</sup> or increased lysolecithin production,<sup>34-3</sup> are emphasized. Without going into any details all these hypotheses do not clearly explain why all the other nonspherocytic hemolytic syndromes (sickle cell anemia, Cooley's anemia, paroxysmal nocturnal hemoglobinuria, etc.) are admittedly not influenced by splenectomy. Survival time studies have shown that the life span of the red cells in normal persons following removal of the spleen remains unchanged.<sup>36-37</sup> It may be argued that this finding does not necessarily invalidate the theories of hemolytic *hypersplenism* because the

other constituents of the reticuloendothelial system substitute for the spleen. In hereditary spherocytosis, however, splenectomy stops permanently the increased rate of hemolysis, and no other organs come apparently into play<sup>36, 37</sup>. Therefore, the reticuloendothelial system does not act as a biologic unit in this syndrome. Consequently the theory of hemolytic hypersplenism could apply only to the spherocytic hemolytic anemias.

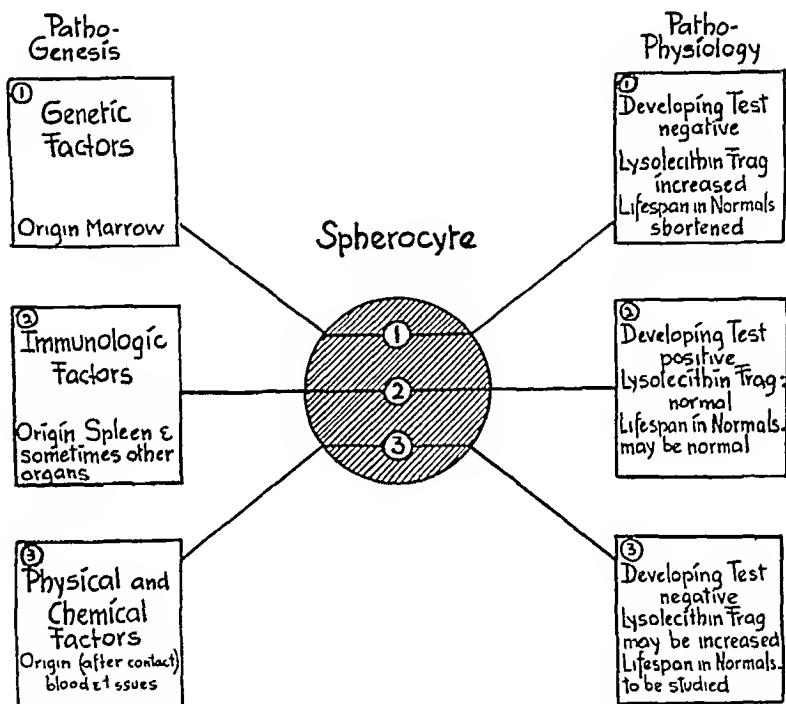


Fig. 1—Differences between morphologically identical spherocytes in clinical conditions

According to the *hypothesis of specific splenic hemolysis*, participation of the spleen in physiologic red cell disintegration is either absent or negligible<sup>39</sup>. The spleen may be the graveyard, but it is not the slaughterhouse of the normal corpuscles<sup>38</sup>. Recent studies have shown that physiologic erythrocyte elimination depends on the age of the cell and also on the mechanical factors to which it is exposed during its life span within the circulation<sup>39, 40</sup>. All hemolytic syndromes are characterized by erythrocytes with specific structural alterations of the cytoskeleton<sup>41</sup>. Such changes may manifest themselves either morphologically or functionally. They may be due to faulty production in the marrow (intra-corpuscular mechanisms) or may develop from the action of external agents on normally constructed erythrocytes (extra-corpuscular mechanisms). In some disorders a combination of both types may be demonstrable, some defective cells may even become vulnerable to physiologic mechanisms which do not attack normal corpuscles<sup>39</sup>.

Splenic hemolysis is predominantly involved in the spherocytic hemolytic syndromes. In the hereditary disorder the spleen selectively removes the un-



coated pathologic cells. In the immunologic acquired variety, the spleen produces the coating antibodies which it is assumed injure the cells and thus render them spherocytic. There is ample experimental evidence available to support these statements. Normal red cells transfused into a patient with familial spherocytosis, have a normal survival time. The patient's uncoated spherocytes, when transfused into a normal person are rapidly destroyed.<sup>8, 20</sup> When spleens, removed for nonhemolytic disorders, are perfused with normal erythrocytes and also with uncoated spherocytes the former pass through the splenic sinusoids whereas the latter are retained.<sup>16, 4</sup> When normal erythrocytes are brought into contact with splenic tissue obtained from patients with acquired spherocytic anemia, the red cells become coated showing distinct agglutination with the anti globulin serum. Control experiments with spleens from patients with hereditary spherocytosis and other conditions fail to give any indication of the presence of an adsorbable globulin.<sup>11</sup>

It is a well known clinical experience that patients with acquired hemolytic anemia may or may not respond to splenectomy whereas patients with the hereditary disorder almost always benefit from this operation.<sup>2</sup> Patients with the acquired disorder may be classified into three groups according to their response to removal of the spleen. In the first group of patients spherocytes disappear completely and permanently.<sup>43</sup> In the second group the coated cells persist pathologic hemolysis stops and these spherocytes exhibit a normal life time in the patient and even when transfused into a normal person.<sup>18</sup> In the third group coated spherocytes continue to be destroyed rapidly. These different responses require an adequate explanation.

In the first group antibody production is apparently restricted to the spleen. This was observed in Patient 8 of our series of acquired hemolytic anemia. Our patient with hereditary spherocytosis with a positive Coombs test also belongs to this category the immunologic mechanism being superimposed upon the genetic anomaly.

The finding of a persistently positive Coombs test after splenectomy indicates that the manufacture of antibodies occurs in other organs also. The fact that splenectomy stops the pathologic hemolysis in some of these patients and does not in others may perhaps be explained on a quantitative basis. If the formation and release of damaging immune bodies proceed on a larger scale in the extra splenic tissues splenectomy will not be successful. However our present understanding of these mechanisms is too incomplete to rule out the possibility that qualitative differences in the coating antibodies and their associated damage to the cytoskeleton may not also be of importance.

The ways and means by which the spleen distinguishes between the different types of spherocytes are not quite clear. Whipple<sup>44</sup> believes that normal discoidal cells have no difficulty in traversing this organ whereas spherocytes being thicker, are unable to pass through the stomata of the splenic sinuses. The demonstration that coated spherocytes when transfused into normals have sometimes a normal survival time<sup>18, 4</sup> renders this hypothesis untenable. It seems however conceivable that the local concentration of antibodies may destroy the cells within the spleen.

other constituents of the reticuloendothelial system substitute for the spleen. In hereditary spherocytosis, however, splenectomy stops permanently the increased rate of hemolysis, and no other organs come apparently into play<sup>36, 37</sup>. Therefore, the reticuloendothelial system does not act as a biologic unit in this syndrome. Consequently the theory of hemolytic hypersplenism could apply only to the spherocytic hemolytic anemias.

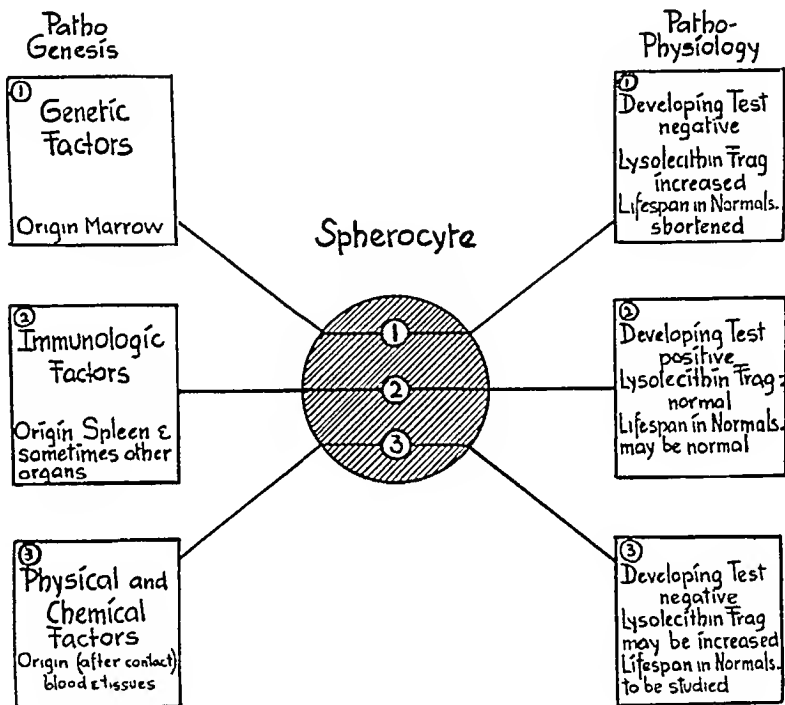


Fig 1—Differences between morphologically identical spherocytes in clinical conditions

According to the *hypothesis of specific splenic hemolysis*, participation of the spleen in physiologic red cell disintegration is either absent or negligible<sup>37</sup>. The spleen may be the graveyard, but it is not the slaughterhouse of the normal corpuscles<sup>38</sup>. Recent studies have shown that physiologic erythrocyte elimination depends on the age of the cell and also on the mechanical factors to which it is exposed during its life span within the circulation<sup>39, 40</sup>. All hemolytic syndromes are characterized by erythrocytes with specific structural alterations of the cytoskeleton<sup>41</sup>. Such changes may manifest themselves either morphologically or functionally. They may be due to faulty production in the marrow (intra-corpuscular mechanisms) or may develop from the action of external agents on normally constructed erythrocytes (extra-corpuscular mechanisms). In some disorders a combination of both types may be demonstrable, some defective cells may even become vulnerable to physiologic mechanisms which do not attack normal corpuscles<sup>39</sup>.

Splenic hemolysis is predominantly involved in the spherocytic hemolytic syndromes. In the hereditary disorder the spleen selectively removes the un-

As may be seen from this discussion our knowledge of the spherocytic hemolytic diseases is still very incomplete. Although the finding of a positive developing test in both the hereditary and the acquired variety diminishes the practical differential diagnostic value of the Coombs test its contribution to the understanding of these syndromes may become significant in the future.

### *B Practical Considerations—*

Any globulin which has become adsorbed onto the surface of the erythrocytes will give a positive developing test with the antiglobulin serum. Since the Coombs test is a nonspecific phenomenon a positive result can be used for differential diagnosis of the spherocytic hemolytic anemia only if any other cause of red cell sensitization has been ruled out. Isoimmunization by the Rh II<sub>1</sub> factors must therefore always be considered in the interpretation of the results. The indirect developing test may be used to exclude this latter mechanism. By incubating the patient's serum with normal O Rh positive (CDE) as well as with O Rh negative (ede) cells and then running the test with the washed erythrocytes one may be able to differentiate between Rh sensitization and the immune bodies operating in the hemolytic anemias. If agglutination occurs with both Rh positive and Rh negative cells Rh antibodies can be excluded. The indirect developing test is based on the assumption that the plasma of the patient contains demonstrable circulating antibodies. However the concentration of these antibodies may be so small that only the sensitive direct Coombs test may betray their adsorption on the red cells. Furthermore, if only third order antibodies are present in the serum, agglutination will not occur even after addition of albumin, but may be detected by means of the developing test.<sup>23, 24</sup> Table V summarizes the results of the procedures to be employed in the differentiation

TABLE V DIFFERENTIATION BETWEEN Rh SENSITIZATION AND IMMUNOLOGIC HEMOLYTIC ANEMIAS

TESTS	Rh SENSITIZATION CIRCULATING ANTIBODIES DEMONSTRABLE	IMMUNOLOGIC HEMOLYTIC ANEMIA	
		WITH	WITHOUT
		CIRCULATING ANTIBODIES	
Direct developing test	Positive	Positive	Positive
Indirect developing test against O Rh positive (CDE) cells	Positive	Positive	Negative
Indirect developing test against O Rh negative (ede) cells	Negative	Positive	Negative

of these two conditions caused by qualitatively different immune bodies. No differentiation between these two states of immunization is possible at present if the patient is Rh negative and is known to have had Rh sensitization. In such cases the Coombs test may be misleading, if not properly evaluated.

"False positive" results<sup>25</sup> may be due to improper technique or to adsorption of globulins caused by nonimmunologic mechanisms. However such conditions, although theoretically conceivable are not known at present. The possible objection that the positive developing test in our case of congenital hemolytic anemia might be a 'false positive' one is disproved by the fact that it was

positive on repeated occasions when the controls were negative and became permanently negative after splenectomy, this patient who was Rh positive never had any transfusions previously—further proof that H<sub>1</sub> immunization could not have played a role either

Not all cells giving a positive developing test have a spherocytic shape. Although spherocytes are sometimes observed in erythroblastosis fetalis,<sup>50, 51</sup> in many cases of this disorder spheroidicity is not demonstrable on the film. Furthermore, cases of acquired hemolytic anemia in adults have been observed where spherocytosis was absent but the Coombs test was positive.<sup>18</sup> Whether coating and alteration in shape can be correlated to specific antibodies requires further studies.

The demonstration of a positive Coombs test in hereditary hemolytic anemia diminishes the value of this test as a means for the differential diagnosis between the congenital and the acquired spherocytic anemias. Since a positive developing test is indicative of an existing immunologic mechanism, a negative test excludes such a condition and speaks strongly for familial spherocytic anemia if drugs and other obvious hemolytic agents can be ruled out. Table VI contains a summary of the disorders in which the performance of the developing test may be of diagnostic value. In our opinion the developing test should become a necessary requirement in diagnosing hemolytic syndromes. If positive, the hemolytic anemia should be designated as being of the "immunologic" type. Such an immunologic hemolytic anemia may even be found superimposed on hereditary spherocytosis.

TABLE VI THE DEVELOPING TEST IN THE HEMOLYTIC ANEMIAS,  
RESULTS OBTAINED SO FAR<sup>10, 18</sup>

POSITIVE DEVELOPING TEST	NEGATIVE DEVELOPING TEST
(A) Acquired spherocytic hemolytic anemias	(A) Hereditary spherocytosis
(1) Idiopathic	(B) Spherocytic anemia due to physical or chemical factors (burns,* sulfa drugs, phenylhydrazine*)
(2) Symptomatic (Hodgkin's disease, leucemia, lymphomas, Boeck's sarcoid, Gaucher's disease,* ovarian tumors,* etc.)	(C) Sick cell anemia
(B) Hereditary spherocytosis (occasionally)	(D) Cooley's anemia
(C) Isoimmunization due to known immune bodies Rh, Hr, A, B, (M*)	(E) Paroxysmal nocturnal hemoglobinuria
(1) Erythroblastosis fetalis	
(2) Sensitization following transfusion	

\*Not actually determined yet but assumed on the basis of other evidence

#### SUMMARY

1 The developing (Coombs) test, using an antihuman globulin rabbit serum, demonstrates the presence of globulin antibodies adsorbed to the surface of erythrocytes. Thus "coated" and "uncoated" red cells can be distinguished.

2 Sixteen cases of spherocytic hemolytic anemia were investigated. All the seven cases of idiopathic acquired type and one case of symptomatic hemolytic anemia (reticulum cell sarcoma) gave a positive test. The result was negative in one patient with hemolytic anemia caused by a sulfa drug. Of the seven patients with hereditary spherocytosis, six showed a negative developing test whereas one severely ill patient displayed a strongly positive reaction.

3 Splenectomy in the acquired cases resulted either in disappearance of the coated spherocytes or the coated cells remained demonstrable, although the pathologic hemolysis ceased

Splenectomy in the case of hereditary spherocytosis with a positive Coombs test led to disappearance of the coating although spherocytosis persisted. This finding demonstrates that two different mechanisms were operating in this particular case

4 A positive developing test indicates the presence of immune bodies. The immune bodies which occur in spherocytic anemias are qualitatively different from the antibodies directed against the Rh H<sub>1</sub> antigens. A method is described to distinguish these two categories of antibodies

5 The existence of "coated" and "uncoated" spherocytes is explained by the hypothesis of the multiple origin of spherocytes. In the hereditary type of hemolytic anemia uncoated cells develop in the marrow under genetic control, whereas in the immunologic variety spherocytes are formed after contact with coating antibodies originating in the spleen and/or other organs. Spherocytosis caused by physical and chemical factors will result from the exposure of normal red cells to these agents either intravascularly or within damaged tissues

6 The theory of specific splenic hemolysis is advanced in order to explain the role of the spleen in spherocytic hemolytic syndromes as contrasted to the current hypotheses of hypersplenism. Participation of the spleen in physiologic red cell destruction is considered negligible. Splenic hemolysis is predominantly involved in the spherocytic hemolytic syndromes. In the hereditary disorder the spleen selectively removes the uncoated pathologic cells. In the immunologic variety the spleen produces the coating antibodies which probably injure the cells and render them spherocytic. Conceivably the cells may then be destroyed within the spleen by an increased concentration of antibodies in this organ

7 The finding of a persistently positive developing test following splenectomy indicates that the production of antibodies may occur in other tissues also. The fact that splenectomy stops the pathologic hemolysis in some patients with acquired hemolytic anemia and does not in others may perhaps be explained on a quantitative basis. If the formation and release of damaging immune bodies proceeds on a larger scale in the extrasplenic tissues splenectomy will not be successful. However, our present understanding of these mechanisms is still incomplete. The possibility exists that qualitative differences in the coating antibodies and their associated damage to the red cells may also be of importance

8 The demonstration of a positive Coombs test in hereditary hemolytic anemia diminishes the value of this test in the differential diagnosis between hereditary and acquired spherocytic anemia. Since a positive developing test is indicative of an existing immunologic mechanism a negative test speaks strongly for familial spherocytosis if drugs and other obvious hemolytic agents can be ruled out

9 The developing test should become a necessary requirement in diagnosing hemolytic syndromes. If positive the hemolytic anemia should be designated as being of the immunologic type. Such an immunologic hemolytic anemia may even be found superimposed on hereditary spherocytosis

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## HYPERTENSION DURING BLOOD TRANSFUSIONS FOR HEMORRHAGIC SHOCK IN A PATIENT WITH UNILATERAL RENAL ISCHEMIA

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DURING the last twenty years the investigations of Goldblatt and many others have firmly established the main outlines of the humoral mechanism of renal hypertension. When renal ischemia occurs from any cause, renin is elaborated in the parenchyma of the kidney and is released into the blood stream. Renin itself has no vasopressor action but it combines with hypertensinogen (renin activator), which is normally present in excess in the plasma and lymph, to produce hypertension (angiotonin). This latter substance has a definite vasopressor effect. A hydrolyzing enzyme, hypertensinase (angiotoninase), is present in various tissues and has the property of destroying or inactivating hypertensin. This agent is found in highest concentration in the intestine, kidney, pancreas, spleen, liver, and erythrocytes. It is present in only small amounts in blood plasma and serum.

Sapirstein, Ogden, and Southard<sup>1</sup> demonstrated a renin-like substance in the blood of dogs during severe hemorrhagic shock. Later they<sup>2</sup> proved that the arterial hypotension associated with extensive loss of blood in the dog was caused partly by exhaustion of renin activator (hypertensinogen). The injection intravenously of relatively small volumes of renin activator, prepared from the globin of ox plasma, would temporarily restore the blood pressure to normal.

Dogs in severe grades of hemorrhagic shock were studied by Collins and Hamilton<sup>3</sup> who found that the renin substrate (hypertensinogen) increased progressively in animals from which the kidneys previously had been removed. In intact dogs there was an initial rise in the concentration of renin substrate which was later followed by a significant reduction below normal as hemorrhage proceeded. They also obtained evidence of increases in the concentration of a substance giving the pharmacologic reactions of angiotonin (hypertensin). Late in the course of shock there was a secondary diminution in angiotonin which was attributed to exhaustion of the renin substrate.

By injecting renin and angiotonin into dogs in hemorrhagic shock, both before and after transfusions of blood were given, Middleton<sup>4</sup> showed that there was little support for the conclusion that there was a deficiency of renin substrate (hypertensinogen). He concluded that the renin-hypertensinogen mechanism was not involved in the occurrence of circulatory failure in shock and that the return of response to renin or hypertensin during circulatory failure remained unexplained.

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The effect of blood transfusions on the blood pressure of human beings in hemorrhagic shock has been observed thousands of times. Although there may be an increase in the amount of renin produced during shock or an exhaustion of hypertensinogen, the transfusion of either fresh or preserved human blood usually does not result in hypertension in the patient in shock. It was therefore of particular interest to encounter a rare combination of circumstances which enabled observations to be made upon a man who received forty blood transfusions in the treatment of hemorrhagic shock. During and after many transfusions the arterial tension rose to abnormally high values for several hours. Autopsy subsequently revealed the presence of a ring of malignant lymphoma around the left renal artery in such a manner as to produce narrowing of the lumen.

#### CASE REPORT

Dr F E W, Hospital No 43 9556, a 53 year old male teacher of veterinary medicine, was examined in the University Hospitals Aug 30 1943, he complained of pain in the lower back and abdomen. In March 1942 he had developed painless swelling of the lymph nodes in the right side of the neck. Biopsy of a lymph node had been diagnosed as lymphoblastic lymphoma and x ray irradiation of the cervical region had resulted in the complete disappearance of palpable nodes in the region. Thereafter he had felt well until a few weeks before admission to the hospital when he experienced diffuse transient pains in the abdomen. He developed severe pain in the lower back which was augmented in the supine position and necessitated sleeping in a chair. The pain became more intense when the spine was hyperextended. There had been no radiation of pain in the distribution of the sciatic nerves. Several days before admission he had noted that the feces became black and his skin slightly pale.

The physical examination revealed a well developed and well nourished man who was not in acute distress. The skin and mucous membranes were pale. There was no cyanosis, dyspnea, or icterus. The pupils reacted to light and accommodation, the ocular fundi were negative. There was no evidence of arteriosclerosis of the retinal vessels. The nose, ears, teeth, and tongue were normal. No lymph nodes or other masses were palpable in the neck. The thorax was symmetrical. The heart was normal in size, rate, and rhythm. No murmurs were heard. The arterial blood pressure measured 130/80. The lungs were normal from percussion and auscultation. The abdominal wall was thick and well muscled. The area of splenic dullness was somewhat increased and a mass descended with inspiration beneath the left costal margin which was thought to be spleen. This was not tender. The liver was not palpable. There was no ascites. The genitalia and rectum were normal. The prostatic gland was not enlarged. Feces removed during digital examination of the rectum were black and gave chemical tests for blood. There was pitting edema of the ankles to the depth of 1 mm to 2 mm. The tendon reflexes were physiologic. There were no areas of tenderness along the spine.

The initial urine specimen examined had a specific gravity of 1.020. It was clear acid and contained no albumin, glucose, or blood. The first blood count showed hemoglobin, 9.0 Gm per 100 ml (Haden-Hauser) erythrocytes 2.57 millions per cubic millimeter, leucocytes 6,750. The differential leucocyte count was 66 per cent polymorphonuclear neutrophils, 1 per cent eosinophils, 1 per cent basophils, 27 per cent lymphocytes, 3 per cent monocytes, and 3 per cent unclassified cells.

An x ray examination of the chest and upper gastrointestinal tract was performed with the aid of a barium meal. The heart and great vessels were normal and there was no enlargement of the mediastinum. The lung fields were clear and the diaphragms were normal in position and function. There was no distortion of the stomach or duodenum although the fluoroscopist thought that the stomach was displaced anteriorly. The colon was examined radiographically with a barium enema but no abnormalities could be found.

The patient was admitted to a hospital bed on Sept 1, 1943. A sigmoidoscopic examination was made the next day. No abnormalities were found except a polyp on the left wall of the rectum which was not bleeding. On September 3 an attempt was made to pass a gastroscope but the procedure was abandoned when the instrument encountered an obstruction at the lower end of the esophagus.

Sept 12, 1943

# Blood Pressure Associated With Blood Transfusions

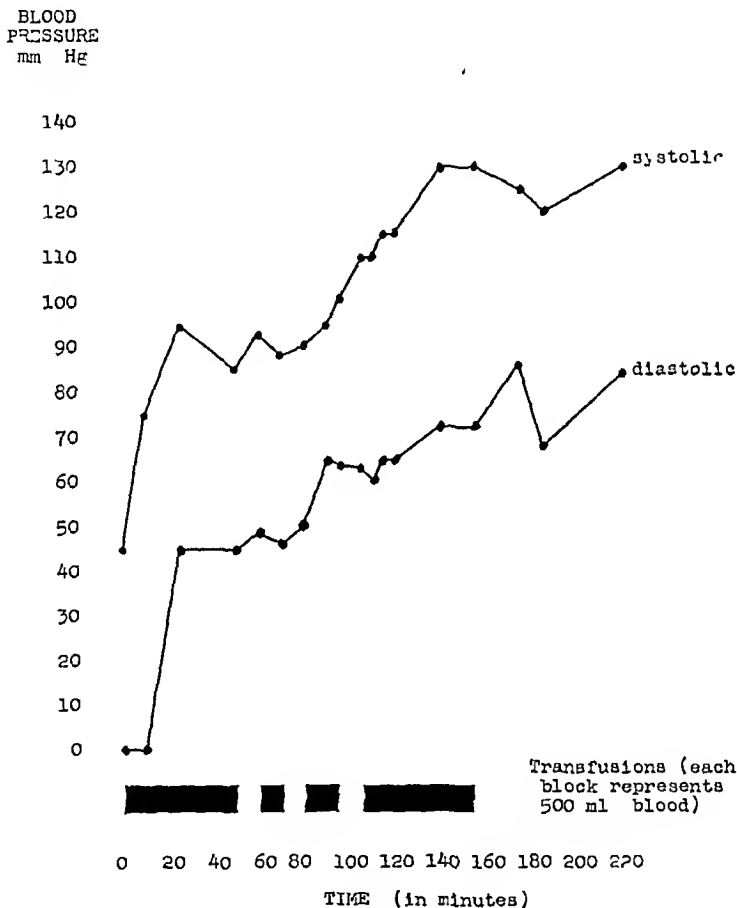


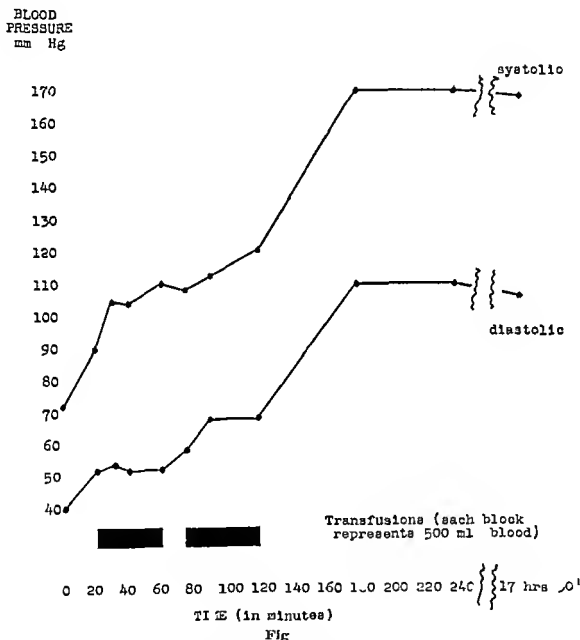
Fig 1

The pain in the back and the upper abdomen had been increasing daily so that sleep was interrupted. X-ray irradiation of the splenic area and epigastrium was begun September 4 and continued until September 10. During that time the patient was given 1,200 r to the retroperitoneal region and 800 r to the epigastrium in daily doses of 200 units on each side. Anorexia increased and the pain in the back and abdomen was augmented. In the evening of September 10 the patient vomited 20 ml of dark blood and passed a small amount of blood per rectum. There was profuse diaphoresis and the volume of the pulse was temporarily diminished. The next day he vomited 150 ml of dark red blood. He was found to belong to group O and was given a transfusion of 500 ml of blood of homologous group preserved in a modified Rous-Turner solution.

At 2350 hours on September 11, without warning the patient vomited a large amount of blood and felt very weak. Thirty minutes later (at 0020 hours September 12) there was another emesis of blood and the passage of a thin firm stool. At 1030 hours there was more vomiting of blood and a feeling of thirst. An hour later the patient suddenly went into profound shock and became unconscious. There had been no abdominal pain but he was under the influence of morphine. Typical Kussmaul breathing developed and the blood pressure

Sept 13 1943

Blood Pressure Associated With Blood Transfusions



measured 40/0. Fig 1 depicts the response of the blood pressure to the transfusion of two liters of preserved blood. Consciousness was recovered at 1430 hours and the patient felt relatively comfortable for the rest of the day. That day the maximum value of the blood pressure after transfusion was 130/84.

On September 13 the patient passed several stools of bright red blood between 1400 and 1600 hours; he sweated moderately and complained of thirst. The blood pressure was 72/40. One liter of preserved blood was transfused with recovery from shock in approximately one and one-half hours. At 1800 hours the blood pressure was found to be 170/110 (see Fig 2). The measurements of arterial tension were checked several times within an hour and found to be correct. At 0830 hours on September 14 the blood pressure was still 168/106. By 1200 hours the tension had declined to 120/80. No blood transfusions were given that day.

The patient again lost large amounts of blood on September 16. There was blood in the vomitus and stools and he went into shock on two separate occasions. The response of the arterial tension to blood transfusions is indicated in Fig 3. With the third transfusion of 500 ml. of blood, the arterial pressure rose from 90/42 to 180/130 in thirty minutes.

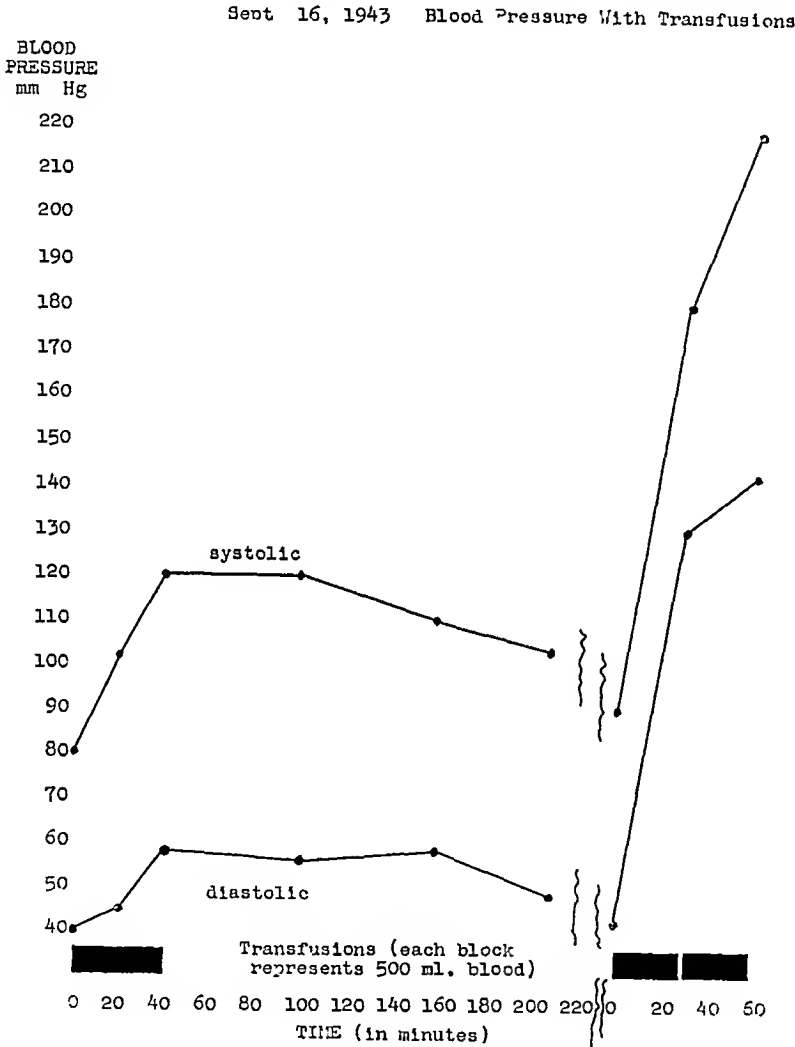


Fig 3

During the fourth transfusion the blood pressure was augmented still further to 218/142 in another thirty minutes.

Fig 4 contains a summary of the transfusions of blood, the daily maximum and minimum blood pressure values, and the erythrocyte counts for most of the hospital stay. It will be noted that on September 19 the blood pressure measured 230/128 although the patient had received no blood transfusions for one and one half days. The next day normal values were observed.

On September 28 the erythrocyte count was 5.3 millions per cubic millimeter, in spite of the fact that there was daily bleeding from the gastrointestinal tract. Beginning September 12 the patient had a continuous low grade fever with rectal temperatures ranging

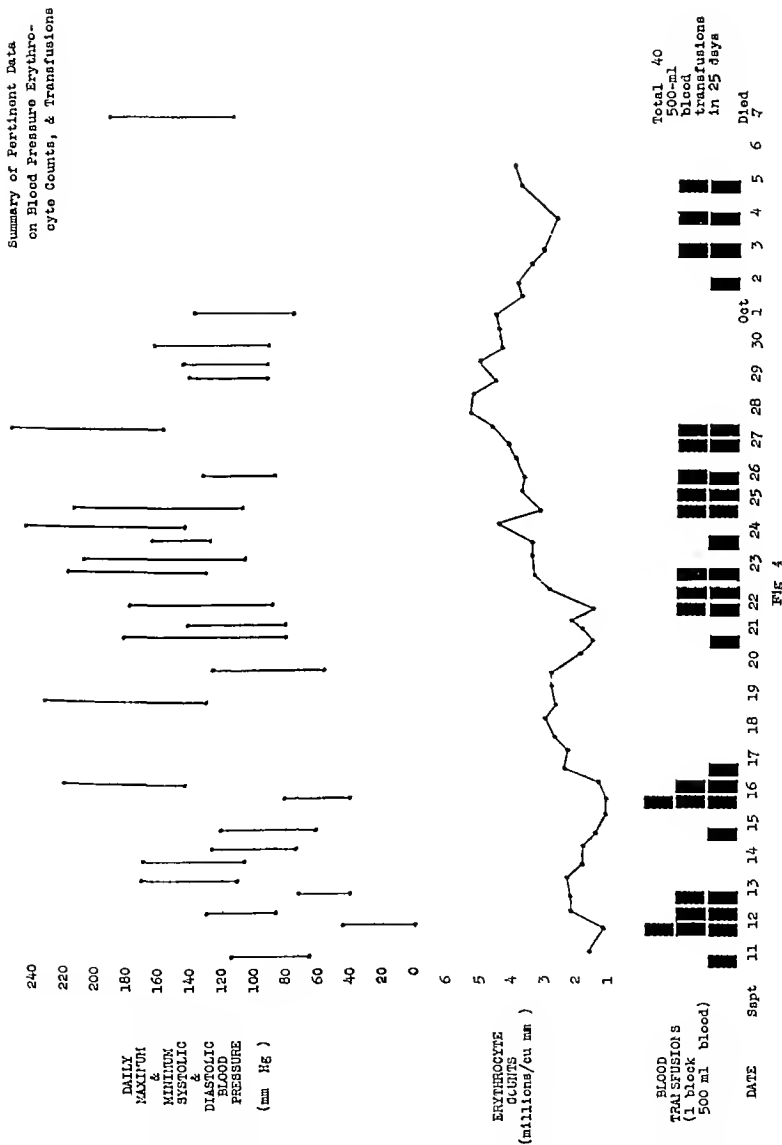


FIG. 4

from 100° to 103° F. On September 20 the blood urea nitrogen was 14.7 mg per 100 ml and the creatinine, 10 milligram. The carbon dioxide combining power of the blood was 59.5 volumes per 100 milliliters. No further blood chemical studies were made. Urine specimens taken after transfusions were negative for hemoglobin. Six urine specimens were examined more completely, the last on September 29. The specific gravity ranged between 1.018 and 1.025. The reaction was always acid. None contained albumin, glucose, or blood. No casts or pus cells were seen in the sediment.

On September 30 the mass in the left upper quadrant of the abdomen was found to have enlarged considerably so that it protruded about four fingerbreadths below the costal margin. It seemed thicker than the spleen should have been with that degree of enlargement. By September 29 the patient was continuously disoriented and complained of terrible pain in the back and abdomen. There was frequent sweating, vomiting of blood, and the passage of bright red blood through the rectum.

On several occasions the patient was questioned concerning measurements of blood pressure before he entered the hospital. He was an eminent scientist who had considerable knowledge of medicine and took a keen interest in the scientific aspects of his condition. He stated that he had been examined several times during the preceding eighteen months at various clinics and the measurements of blood pressure always had been reported as normal.

TABLE I DATA ON THE DONORS AND THE STORAGE OF THEIR BLOOD FOR TRANSFUSION  
(ALL BELONGED TO GROUP O AND EACH TRANSFUSION WAS WITH 500 ML OF BLOOD)

DATE OF TRANSFUSION	AGE OF DONOR	DAYS OF STORAGE OF BLOOD	HYPERTENSIVE EFFECT	DATE OF TRANSFUSION	AGE OF DONOR	DAYS OF STORAGE OF BLOOD	HYPERTENSIVE EFFECT
Sept 11	31	17 R T	Absent	Sept 23	24	1 C	Present
Sept 12	53	18 R T	Absent		25	2 C	Present
	47	18 R T		Sept 24	35	13 R T	
	27	6 C		Sept 25	59	8 R T	Present
	25	6 C			33	6 R T	
	21	4 C	Present		51	2 C	Absent
Sept 13	33	5 C			21	2 C	
	40	4 C		Sept 26	21	6 R T	Present
Sept 15	30	3 C	Absent		33	7 R T	
Sept 16	39	17 R T	Present	Sept 27	27	7 R T	Present
	24	3 C			28	0 C	
	26	2 C			27	0 C	
	34	2 C			26	0 C	
	44	14 R T	Not observed	Oct 2	54	4 C	Not observed
Sept 17	28	14 R T		Oct 3	26	6 C	Not observed
Sept 21	20	4 C	Present		23	6 C	Not observed
Sept 22	39	3 C	Present	Oct 4	32	6 C	
	20	4 C			26	0 C	Not observed
	41	3 C		Oct 5	42	2 C	
	33	3 C			41	2 C	

R-T Blood preserved in modified Rous-Turner Solution

C Blood stored in sodium citrate solution

Altogether the patient received forty blood transfusions of 500 ml each. The blood was preserved in either a modified Rous-Turner solution or it was collected in sodium citrate solution and given within a few hours or days. The patient belonged to group O and, of course, received only blood of that group. Typing of the Rh antigen was not performed. There was one questionable hemolytic reaction after transfusion, all others were uncomplicated. The data on the donors whose blood the patient received are noted in Table I. Blood pressure measurements were not made on the donors.

## POST MORTEM FINDINGS

A complete autopsy was begun one hour and forty minutes after death \*

The body was that of a well developed and well nourished man 166 cm in length and weighing 79.5 kilograms (175 pounds). There were no skin lesions or edema. The superficial lymph nodes were not enlarged.

*Peritoneal Cavity*—Before an incision was made a large mass could be seen and felt which occupied the left upper quadrant of the abdomen and the epigastric region. Section of the abdominal wall revealed good musculature and a layer of adipose tissue about 1.5 cm in thickness. Inspection of the peritoneal cavity disclosed a large nodular mass which occupied the left upper quadrant. It was firm and white. It involved the entire stomach, pancreas, pleural left adrenal gland and left kidney, the aorta and vena cava with their associated vessels, and retroperitoneal lymph node. The mesentery of the transverse colon and the splenic flexure was adherent to the mass but there was no constriction of the lumen of the bowel. The small intestine, right adrenal and right kidney were not involved. Some white strings of tumor tissue extended up into the hilus of the liver.

When the stomach was opened the crater of an ulcer the size of a man's fist was revealed in the posterior wall of the fundus. The ulcer had penetrated the spleen and excavated a cavity about 10 cm in diameter and 5 cm deep. The base of the ulcer lay deep in the spleen so that blood had passed freely from the splenic pulp into the stomach.

The retroperitoneal lymph nodes were enlarged to measure between 3 cm and 6 cm in diameter. They were soft white and cellular. They surrounded the aorta, vena cava, left renal vessels, the aortic arch and the superior mesenteric vessels. The pancreas was completely surrounded by tumor.

Microscopically it was seen that the normal architectural pattern had been completely replaced by a malignant neoplasm of the lymphoma group. The tumor for the most part, consisted of masses of lymphoblastic cells with a monotonously uniform pattern since there were no attempts at follicle formation. The lymphoblasts were round or oval and rather pale-staining. Scattered among the cells however, were numerous reticulohistiocytes in various stages of maturation. Many showed phagocytic activity. Scattered through the neoplastic tissue were several clumps of plasma cells. In other areas mononuclear cells probably monocytes were seen arising from the reticulum stroma particularly where there was hyperplasia of the reticuloendothelial elements. Occasionally there occurred rather large collections of the so-called syncytial forms of primitive reticuloendothelial cells. These had dense masses of chromatin within the nuclei and only a slight amount of cytoplasm. These cells were arranged in cords and strands which separated the connective tissue stroma. Despite the various cell types present the lymphoblasts obviously predominated and it was apparent that the tumor was fundamentally of reticuloendothelial origin in which maturation had been arrested at the lymphoblastic stage. However, occasional clusters of immature lymphocytes also were observed which indicated that further maturation had occurred in some regions.

Many blood vessels particularly in the spleen were filled with neoplastic cells so that tumor thrombi were conspicuous. Numerous normal and pathologic mitotic figures were observed. In general the tumor appeared to be growing rapidly and was highly invasive. Local areas of necrosis especially in the spleen were found of which some were hemorrhagic from early infarction and others appeared older.

*Thoracic Cavity*—The left hemithorax contained 2900 ml of seroanguineous fluid. There were 100 ml of clear yellow fluid in the right hemithorax. The left lung was collapsed and the right was somewhat emphysematous.

*Medastinum*—Just above the bifurcation of the trachea was a white cellular lymph node about 4 cm in diameter. Strings of white friable tumor tissue descended from this through the diaphragm and were interpreted to be lymphatic trunks filled with neoplasm.

\*The pertinent gross findings were reported by Dr. C. J. Mikkelsen while I am indebted to Dr. J. N. Cutler of the Department of Pathology for the microscopic findings.

*Heart*—The pericardial walls were smooth and glistening. The heart weighed 330 grams. The chambers were not dilated. The wall of the left ventricle was 1.5 cm in thickness, the right was 0.5 centimeter. The valve rings measured: aortic, 7.5 cm, pulmonary, 7.8 cm, mitral, 10.0 cm, and tricuspid, 11.8 centimeters. The foramen ovale was closed. The epicardium, endocardium, columnae carneae, papillary muscles, chordae tendineae, heart valves, and coronary vessels were normal.

Microscopic examination showed the epicardium to be thin and the epicardial fat abundant. There was no evidence of inflammation. The myocardium appeared virtually normal. There was no evidence of hypertrophy, infection, or infarction.

*Lungs*—The right lung weighed 400 grams. It was pale and the alveoli were slightly larger than normal. No lesions were found on section. The left lung weighed 420 grams. It was red, had the consistency of soft rubber, and sank when immersed in water. When sectioned, a red infarct about 8 mm in diameter was seen which contained one small, soft, yellow area.

Sections viewed microscopically showed focal aggregates of neoplastic cells in and beneath the pleura. The malignant cells extended a moderate distance into the parenchyma of the lung. Both arteries and veins were invaded by tumor cells. Focal atelectases were observed and adjacent to some were regions of compensatory emphysema. There was mild but definite thickening of the pulmonary arteries and arterioles. In one locus the parenchyma of the lung had been replaced by a mass of necrotic debris and hemorrhage. This area was well circumscribed and appeared consistent with an infarct about twelve hours old.

*Spleen*—The spleen could not be weighed accurately because it was embedded completely in neoplasm and surrounded by adherent omentum, diaphragm, and body wall. It measured about 15 by 20 centimeters. The upper medial portion formed a dark base for the ulcer in the stomach and the excavation was the size of a half grapefruit. Sections showed firm yellow areas in the parenchyma which were a maximum of 8 mm in diameter. Similar areas were barely visible to the naked eye. Very little red pulp was seen. No fresh infarcts were observed. The blood vessels at the base of the ulcer were patent and gaping, some of the lumens being 1 mm to 2 mm in diameter. The microscopic appearance is described under Peritoneal Cavity.

*Gastrointestinal Tract*—The esophagus was normal, but just above its entrance to the cardia of the stomach a neoplastic lymph node about 10 cm in diameter was situated anterior to the vertebral column so that it probably obstructed the passage of the gastroscope. Large masses of retroperitoneal nodes were filled with neoplasm in such a manner as to displace the stomach anteriorly. The upper half of the posterior wall of the stomach was completely adherent to the mass and to the spleen. In the posterior wall of the gastric fundus was the mouth of the ulcer which communicated with the interior of the spleen. This orifice was about 12 cm in diameter. The wall of the ulcer was so thin that soon it would have ruptured into the lesser omental sac.

The small intestine, colon, appendix, and rectum were normal except that the transverse and splenic portions of the colon were adherent to the spleen, although they were not invaded by neoplasm.

*Liver*—The liver weighed 1,900 grams. Section of the pedicle revealed white streaks extending into the parenchyma and yellow discrete areas measuring from 1 mm to 4 mm in diameter.

Microscopically much of the parenchyma appeared to have been replaced by masses of malignant cells. For the most part, the largest aggregates of neoplasm were in the portal areas. However, contiguous and ramifying masses of tumor cells involved the central and the subzonal portions of the lobules. Mild fatty metamorphosis was present and there was active regeneration of liver cells in some areas, as evidenced by enlarged hyperchromic and frequently double nuclei. Small regions of necrosis were present.

*Pancreas*—This gland was surrounded by neoplastic tissue but microscopically the general architectural pattern seemed to be preserved. In several areas there was invasion of the gland by neoplastic cells, principally along the interlobar and interlobular septa. Small focal necroses had occurred and one zone of fat necrosis was seen. Invasion of the blood vessels by neoplasm was observed.



**Kidneys.**—The left kidney weighed 150 grams. The parenchyma appeared normal to the naked eye. The hilus was embedded in a mass of neoplasm which formed a complete ring around the renal artery. Longitudinal section of the vessel showed it to be patent but it was apparent that the lumen was narrowed by compression of the constricting ring of tumor (see Fig. 5).



Fig. 5—Photograph showing section of abdominal aorta and left renal artery laid open. On extreme right is the hilus of the left kidney. The renal artery is completely surrounded by a ring of lymphomatous tissue so that its lumen is compressed. (Photograph lightly retouched.)

The right kidney weighed 150 grams. Under the capsule were two yellow wedge shaped areas about 0.5 cm. in diameter. The parenchyma was otherwise normal.

Microscopically the architecture of both kidneys seemed to be well preserved. A few hyalinized and fibrotic glomeruli were present but most were within normal limit. Although there were a few degenerated tubules most were virtually normal. There was no evidence of lower nephron nephrosis or nephritis. Many of the tubules however contained precipitated protein and granular debris. The interstitial tissue was not edematous and the vessels in the parenchyma were normal. The capsule and superficial cortical tissue of the left kidney was invaded by solid masses of malignant cells. Large areas of necrosis were present in the neoplastic tissue. In the right kidney was seen a recent infarct which appeared to be about forty-eight to seventy-two hours old. Surrounding this there was a conspicuous zone of hyperemia and acute cellulitis.

Both pelves and ureters were normal and patent.

**Adrenals.**—The right adrenal gland appeared normal both grossly and microscopically. The left was fixed to the tumor tissue. In the microscope the capsule was seen to be invaded and replaced by masses of neoplasm. In a few regions there was also beginning invasion of the superficial portion of the cortex. For the most part however the cortex was well preserved. Foci of congestion were present in the medulla.

*Blood Vessels*—A few atheromatous plaques were seen in the aorta. In the upper abdomen the vessel was surrounded by neoplasm but there was no encroachment on the lumen. The greater portion of the left renal artery was surrounded by a thick collar of neoplastic cells, as seen macroscopically. The cells had invaded and replaced the adventitia and were beginning to penetrate the media. For the most part, the neoplastic involvement was superficial. In some areas the intima was slightly thickened and the subintimal connective tissue was somewhat diminished. No thrombi were present.

*Diaphragm*—Microscopic sections showed invasion of the muscle bundles by strands and small masses of neoplastic cells.

*Brain, Genitalia, Gall Bladder, and Urinary Bladder*—These were entirely normal.

#### *Anatomic Diagnoses*—

Malignant lymphoma, mixed type, predominantly lymphoblastic, involving the retroperitoneal lymph nodes, spleen, liver, stomach, left kidney, left adrenal, pleura, and lung.

Massive ulceration of stomach into spleen with hemorrhage.

Recent infarcts of lungs, spleen, and kidneys.

Serosanguineous pleural effusion, left.

Partial collapse, left lung.

Compensatory emphysema, right lung.

Arteriosclerosis, generalized.

#### DISCUSSION

There can be little doubt that the patient did not have hypertension before the blood transfusions were given in the hospital. Several measurements of arterial tension were made before the onset of the severe hemorrhage by me and other members of the hospital staff and all values were found to be within the normal range. The patient had had several examinations by competent physicians in the eighteen months before admission to the hospital and hypertension had not been noted. Furthermore, the anatomic examination of the heart and blood vessels support the conclusion that essential hypertension was not a part of the clinical picture. The myocardium was not hypertrophied and the chambers of the heart were not dilated. There were few atheromata in the walls of the aorta. The coronary vessels appeared normal. There was little sclerosis of the smaller vessels.

All available evidence points to the conclusion that the transient episodes of arterial hypertension in this patient were caused by (1) the transfusions of blood, (2) the severe hemorrhage and shock caused by the erosion of splenic vessels with lumens of 1 mm. to 2 mm. in diameter and the loss of blood directly into the stomach, (3) the ischemia of the left kidney whose artery was constricted by a ring of neoplasm, or (4) a combination of two or more of the three factors.

It is probable that preserved human blood contains some hypertensinogen, rennin, and hypertensin. Kohlstaedt, Page, and Helmer<sup>6</sup> noted that rennin activator (hypertensinogen) disappeared in five or six hours from dog's blood kept at room temperature. It was present in similar blood stored at 10° C. for a week. However, blood transfusions have not been observed to produce hypertension in the usual clinical experience. The blood pressure is measured frequently in patients in hemorrhagic shock who receive blood transfusions as the principal method of treatment. Yet hypertension is not usually associated

with recovery from shock. If the experimental studies previously quoted can be applied to human beings in shock it would seem that the loss of hypertensinogen by hemorrhage and the secretion of renin by the kidneys whose circulation is impaired are quantitatively not sufficient to produce an augmentation of arterial pressure above the normal when blood transfusions are given.

The unusual anatomic feature of the case under discussion was the compression of the left renal artery by neoplasm. Renal ischemia in comparable circumstances has been shown repeatedly to produce hypertension under experimental conditions. It remains to relate this to the facts which are known about transfusion and hemorrhagic shock. It is conceivable that if some renin is produced by the otherwise normal kidney when the organism undergoes severe hemorrhagic shock, more renin might be produced with the same degree of circulatory failure if the renal artery were already constricted. Although experimental evidence is not at hand, it seems that this much must be assumed to explain the facts in this case.

Two alternatives are possible to explain the association of the hypertension with blood transfusion: (1) that the transfusions were merely coincidental, or (2) that the transfused blood replaced hypertensinogen lost in hemorrhage so that the increased amount of renin was furnished with substrate to produce hypertension.

Since in each instance of hemorrhagic shock in this patient blood transfusions were given, it does not seem possible from the data presented to choose between the alternatives of coincidence and the replacement of hypertensinogen. It cannot even be assumed that the patient was not in shock when the blood pressure was at hypertensive levels because Middleton<sup>4</sup> demonstrated that renin or hypertension had vasopressor effects in the shocked dog even in the presence of circulatory failure. In the light of his work the fact that some transfusions were not associated with hypertension in the patient cannot be construed as evidence that the donor's blood lacked hypertensinogen or that the patient's kidney was not producing an increased amount of renin. Middleton found that dogs in shock responded to renin and hypertension at times but not at others and concluded that the cause of the irregularity was not yet elucidated.

#### SUMMARY

Observations on a patient with malignant lymphoma are recorded. Eroded blood vessels in the spleen led to extensive blood loss through a communicating ulcer in the stomach. A ring of neoplastic tissue surrounded and compressed the left renal artery. During a period of twenty-five days the patient received forty 500 ml blood transfusions after many of which the blood pressure ascended to hypertensive levels for many hours. It is concluded that the combination of hemorrhagic shock and renal ischemia from renal artery compression caused an unusual increase in the output of renin. No conclusion could be reached as to whether the occurrence of the hypertension after blood transfusion was coincidence or whether the donor's blood supplied hypertensinogen which had been exhausted by hemorrhage. Only experimental studies on animals can completely elucidate this point.

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# THE EFFECT OF HEPARIN AND DICUMAROL IN INCREASING THE CORONARY FLOW VOLUME

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## INTRODUCTION

**D**URING the past several years considerable interest has arisen in the results obtained in the treatment of coronary thrombosis with anticoagulant drugs. Several series of cases have been reported and important work has been carried out showing reduction of mortality and decrease in incidence of thromboembolic phenomena. This work has been amply reviewed by several authors, among whom are Wright, Marple, and Beck,<sup>1</sup> Nichol and Page, Parker and Barker.<sup>2</sup>

The results obtained with Dicumarol and heparin so closely approximate those which we have obtained with other coronary vasodilator drugs, such as the xanthine series, that the question arises whether or not the results obtained with the anticoagulant drugs are not due to some effect other than their anticoagulant action. It did not seem altogether reasonable that the lower mortality and fewer embolic accidents now being reported in patients treated with heparin and Dicumarol were due to the anticoagulant action alone.

When the patient is seen by the physician, the occlusion and the infarct are already an accomplished fact. Anticoagulant treatment cannot possibly alter the already formed thrombus or have any effect upon the infarct itself whatever effect it might have on future thrombotic processes. There is no definite evidence that the thrombus propagates or extends and there is a great deal of evidence that the thrombus does not propagate. Fooid,<sup>3</sup> who has made a careful study of thrombi occurring in coronary vessels states that when the major coronary vessels are dissected out in toto fixed in formalin, and decalcified the length of the thrombus in the artery, in almost all cases is a matter of only a few millimeters, and only occasionally is a thrombosed portion found to be 1 cm in length in the right coronary artery. Another factor to be considered in the use of anticoagulants is that many cases of occlusion are initiated by subintimal hemorrhage. Wartman,<sup>4</sup> in a careful study of occluded coronary arteries found 14.6 per cent occluded solely by intimal hematoma, while an additional 34.2 per cent showed a combination of intimal hemorrhage and thrombosis. It is his current impression that subintimal hemorrhage is a rather common occurrence and can be demonstrated if especially looked for. Dolos<sup>5</sup> has reported treating acute coronary occlusions with vitamin K with good results.

Nichol<sup>7</sup> in discussing a paper by Falk<sup>6</sup> as well as his own results with Dicumarol therapy in patients with coronary thrombosis stated that congestive failure of a severe degree is less frequent when Dicumarol is used. He also cites a number of patients with repeated coronary occlusions who when placed on

daily continued doses of Dicumarol, showed no evidence of coronary insufficiency. When reported, these patients were under observation for periods ranging from six to thirty-three months. Coogan<sup>9</sup> reports a similar experience with Dicumarol in the ambulatory management of six patients suffering from angina pectoris. This has been further confirmed by our own clinical observations of patients with coronary insufficiency who were treated with Dicumarol. It is hardly to be assumed that relief of pain in angina pectoris would be due to any anticoagulant action. It is much more reasonable to assume that relief is due to the coronary vasodilating action increasing the coronary flow.

Cotlove and Voizimer,<sup>10</sup> in making serial prothrombin estimations in cardiac patients, concluded that little is known of the relation of the prothrombin activity of the blood to the occurrence of intravascular thrombosis. They state that there is no evidence that an increase in prothrombin activity leads to thrombosis and that perhaps the abnormal acceleration of the dilute plasma prothrombin time which has been observed in cases of thromboembolism may be entirely secondary to the thrombosis already formed. This study is substantiated by the work of Cummine and Lyons.<sup>11</sup>

In reviewing the clinical reports of coronary thrombosis receiving Dicumarol treatment, we felt that perhaps some additional factor was responsible for the unquestioned, improved results. We therefore instituted a series of experiments upon animals and upon the empty beating heart. We have been able to show that the use of heparin or Dicumarol increases the coronary flow volume. With heparin, the effect varies from no effect to moderate, depending upon the preparation. Dicumarol, however, has a very definite and marked effect in increasing the coronary flow. The Dicumarol effect is equal to that of the xanthines and is of apparently longer duration.

#### MATERIALS AND METHODS

Healthy mongrel dogs of either sex were anesthetized with Dial and urethane, and under positive endotracheal oxygen pressure, the chest was opened parasternally on the right. The right auricular appendage was nicked, and a modified Morawitz Zahn cannula was inserted into the coronary sinus. This technique for measuring coronary flow is simple. It requires only a matter of three to five minutes to insert the cannula and for the blood to appear in the measuring cylinder. There is a minimum of trauma and the reflexes are not disturbed. The authors recognize that this method does not measure total coronary flow, but it does properly show variations in flow. For this purpose, we think this method better than more elaborate methods in which greater trauma is incurred.

During the heparin experiments, hirudin was used as an anticoagulant. During the Dicumarol experiments either hirudin or a heparin which was known to have no vasodilator effect was used. Blood pressure recordings were made, using a mercury manometer connected to a cannula inserted into a carotid artery. This method was considered adequate for these experiments. Empty beating heart experiments were performed, in which the dogs' own blood was used for perfusing the coronary vessels, in order to show the changes in total coronary flow.

#### RESULTS

An aqueous solution of theobromine sodium acetate, freshly prepared each day, was used as a standard control in the experiments. If, after the intra

venous injection of this drug a prompt rise in coronary flow followed, it was considered that the apparatus was functioning properly. Fig 1 illustrates a typical coronary vasodilator response to the theobromine. Table I shows the results of a single dose of theobromine on the coronary flow.

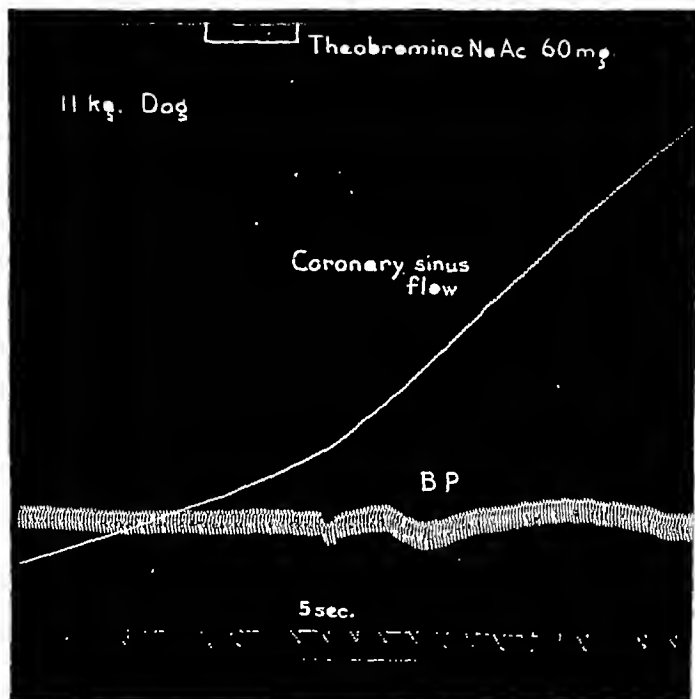


Fig 1—This tracing illustrates the usual increase in coronary sinus flow following an injection of theobromine sodium acetate.

TABLE I

	BEFORE	20 SECONDS LATER	% CHANGE
Coronary flow (ml per min.)	189	530	180.0 plus
Pulse	100	104	4.0 plus
Blood pressure (mm mercury)	12	74	2.8 plus

Dog 3 60.0 mg theobromine sodium acetate given I.V.

Following the same technique as with theobromine, heparin was used. As mentioned earlier, the effect with heparin varied from no effect to moderate, depending upon the preparation used. The sodium salt of heparin, which is used clinically, showed slight to moderate coronary vasodilator effect. The barium salt of heparin did not alter the rate of coronary flow, on repeated

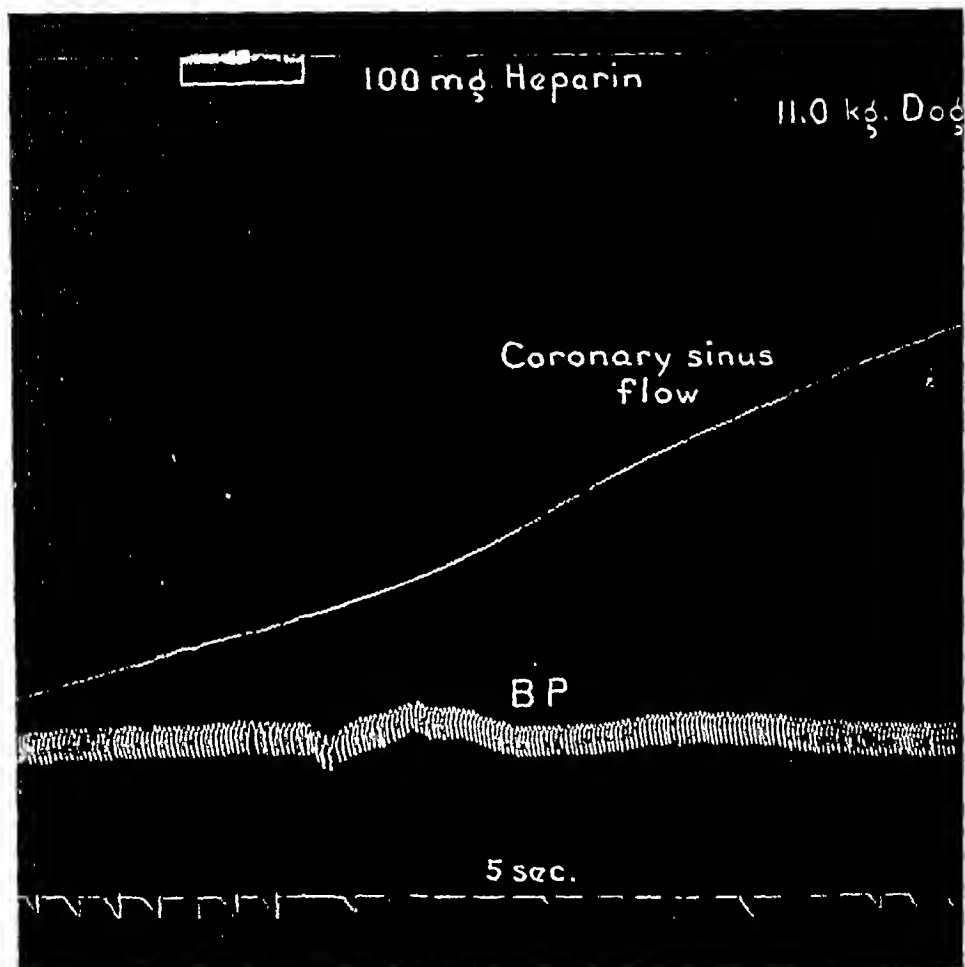


Fig. 2—Following the administration of heparin an increase in coronary sinus flow follows with little associated change in blood pressure and pulse

trials, at various dosages. It has been our experience that soluble barium salts have a vasoconstricting action upon the coronaries<sup>12</sup>. It is reasonable to assume that the vasoconstricting action of the barium ion in the heparin preparation neutralizes the dilator effect of heparin. Fig. 2 and Table II illustrate the increase in coronary flow following intravenous administration of sodium heparin. The effect is not as marked as with the xanthines, but does show an increase in coronary flow of 77.6 per cent, with a fall in blood pressure of 2.7 per cent, and an increase in pulse rate of 4.2 per cent.



TABLE II

	BEFORE	20 SECONDS LATER	% CHANGE
Coronary flow (ml per min )	170	302	77.6 plus
Pulse	96	100	4.2 plus
Blood pressure (mm. mercury)	74	72	2.7 minus

Dog " 100 mg heparin given I V

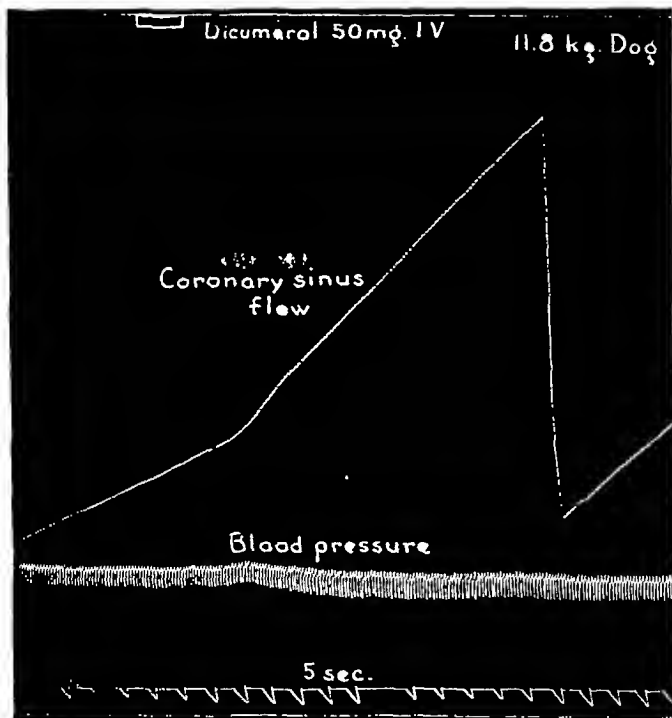


Fig 3—That Dicumarol is a strong coronary vasodilator is shown by this kymograph tracing. The increase in coronary sinus flow following Dicumarol appeared to be of longer duration than that produced by xanthines.

In the Dicumarol experiments the disodium salt of Dicumarol was prepared from a Dicumarol base of high purity, melting at 288 to 289° C. This was diluted with physiologic saline solution and given intravenously. The effect of Dicumarol on coronary flow is illustrated in Fig 3 and in Table III. This type

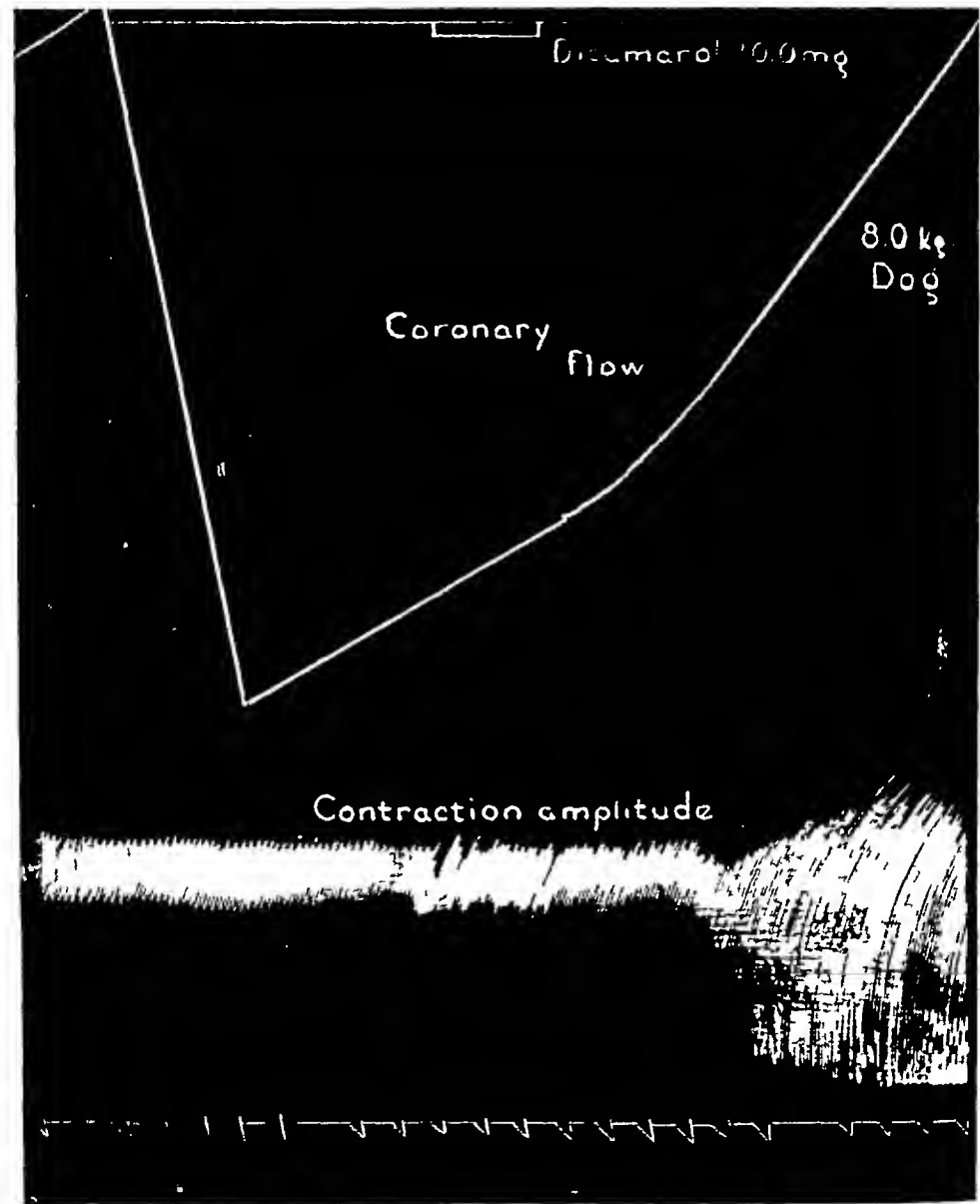


Fig 5 —In the isolated heart experiment the total coronary flow shows a sharp increase in the rate of flow following the addition of Dicumarol to the perfusing blood

TABLE V

	BEFORE	20 SECONDS LATER*	% CHANGE
Coronary flow (ml per min )	85 0	179	111 0 plus
Pulse	128	148	15 6 plus

\*Dog 26 10 0 mg Dicumarol given

observation of profound capillary dilatation that follows administration of dicoumarin, even when only moderate doses are given, raises the question of a gross vascular factor in the hemorrhage manifestation of sweet clover disease in cattle." Bollman and Preston<sup>17</sup> report similar results, and Dale and Jaques<sup>18</sup> also state that they have confirmed the results of Bingham and his group

# SUMMARY

We have shown in these experiments that heparin and Dicumarol increase the coronary flow volume. We believe that the beneficial results of these drugs in coronary thrombosis and in angina pectoris are due to the increase in coronary flow which accompanies their use.

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# THE ONE-STAGE AND TWO-STAGE PROTHROMBIN METHODS IN THE CONTROL OF DICUMAROL THERAPY, WITH REMARKS ON Ac-GLOBULIN

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## PURPOSE

THE indications for the administration of Dicumarol have broadened in recent years, yet the effective methods for its control have not materially changed. Efforts have, for the most part, been directed toward developing a uniformly reactive thromboplastin for use in the one stage method for prothrombin determination, or toward altering the original one-stage procedure as described by Quick<sup>12</sup> to render it more uniform or more sensitive in reflecting prothrombin variations. This paper deals with comparative results of the one-stage and two-stage prothrombin methods on the blood of dicumarolized patients. In addition, early experience with the behavior of the Ac-globulin in dicumarolized patients is reported and suggestions are offered as to the possible relationship of this factor to disparities between the one-stage and two stage methods.

## GENERAL STATEMENT

In the one-stage prothrombin method as described by Quick,<sup>12</sup> thromboplastin, calcium, and temperature are controlled factors. Possible variables, including antithrombin,<sup>10</sup> antithromboplastin,<sup>13</sup> fibrinogen,<sup>5, 9, 16</sup> heparin,<sup>1, 2</sup> and the rate at which prothrombin is converted to thrombin,<sup>14, 17, 18, 20, 21</sup> are not controlled. The catalytic action of heparin on antithrombin<sup>15</sup> also may be a disturbing factor. In the two stage method, dilution of the plasma materially reduces the activity of the antithrombin<sup>22</sup> and antithromboplastin.<sup>3</sup> Standardized fibrinogen solution is added in the two stage test, and the two stage principle, with the addition of Ac-globulin, eliminates as a variable the conversion rate of prothrombin. Heparin may be a variable in either test and in sufficient concentration will influence the results.<sup>3</sup>

## METHODS AND PROCEDURE

This report is based on the study of 140 patients on Dicumarol therapy.

In the first ninety nine cases, the two stage prothrombin method, as devised by Warner, Brinkhous, and Smith,<sup>22</sup> was used throughout as the guide in administering Dicumarol. In the succeeding forty one cases, the Ac globulin factor was added according to the modification of Seegers.<sup>5</sup> The former procedure will be referred to in this paper as the unmodified two stage method, and the latter as the modified two stage method.

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In ninety five of the first ninety nine cases the one stage test as described by Quick<sup>12</sup> was run in parallel\* to the two stage determination. In the other cases it was run at frequent intervals, usually initially and nearly always when the two stage method gave a reading below 10 per cent of normal. In all instances the thromboplastin for the one stage test prepared from acetone dried rabbit brain met Quick's requirements for this reagent.

In ten of the first ninety nine cases the Quick test was run on plasma diluted to 12.5 per cent of normal according to the method of Link<sup>4</sup> and associates. In these, a per cent plasma was also used.

In eighteen patients, Aeglobulin determinations were made according to the method of Ware and Seegers.<sup>13</sup> Blood was drawn by venepuncture into 3.2 per cent sodium citrate (13 volumes to 2 volumes respectively) and processed as previously described.<sup>6</sup> In seven of these the determinations were made from the beginning of the therapy, and in addition, the one stage and two stage prothrombin assays were made both with and without the addition of Aeglobulin. In the case of the one stage test, the Aeglobulin was added by mixing 0.1 cc of beef serum, a potent source of the factor<sup>13</sup> to 0.9 cc of whole human plasma. The procedure described by Quick<sup>12</sup> will be referred to as the unmodified one stage method, and the same procedure using 10 per cent beef serum will be designated as the modified one-stage method.

Original doses of Dicumarol varied between 200 and 500 mg depending on the original prothrombin level and the size of the patient. The following day 100 to 200 mg were given, and the succeeding doses were varied according to the prevailing prothrombin level. After proper stabilization prothrombin determinations were made at weekly intervals, and in some cases every two weeks. If bleeding occurred, Dicumarol was stopped or the dosage reduced. In three instances vitamin K was given intravenously in doses of 24 mg at intervals of two hours until the prothrombin rose to nonbleeding levels. Periods of therapy varied up to a maximum of two years with an average of sixty days.

## RESULTS

In all cases the two stage method with or without Aeglobulin added, gave more uniform readings and provided for a more accurate maintenance of the prothrombin level (Fig 1). In most instances it was possible to maintain the prothrombin within a bracket of 5 to 35 per cent of normal with an occasional reading above or below these extremes. With the unmodified two stage test the ideal prothrombin range was 10 to 20 per cent. When the modified two stage procedure was used the ideal range was 20 to 30 per cent.

The one stage method in some instances gave results parallel to and usually higher than those of the two stage test but in most cases it varied widely from day to day (Fig 1). The one stage technique using 12.5 per cent and 5 per cent plasma likewise gave widely varying results (Fig 2). Bleeding occurred in 10.7 per cent of the patients. This usually consisted of hematuria, gross or microscopic. A few patients showed subcutaneous hemorrhage or bleeding from the gastrointestinal tract. There were no instances of serious bleeding. Of nine patients on therapy during active menstruation, two bled longer and more profusely than ordinarily. The others noticed no variation from the usual period.

In the seven patients on whom Aeglobulin determinations were made from the beginning of therapy, this factor dropped in the first few days and

\* Occasionally owing to technical or personnel difficulties a test, either one stage or two stage was omitted.

returned toward normal by the end of twenty-one days (Fig 3) The lowest level recorded was 28 per cent of normal In eleven patients who had been on Dicumaiol therapy for a period of three weeks or longer, some as long as eighteen months, there was no consistent variation from the normal

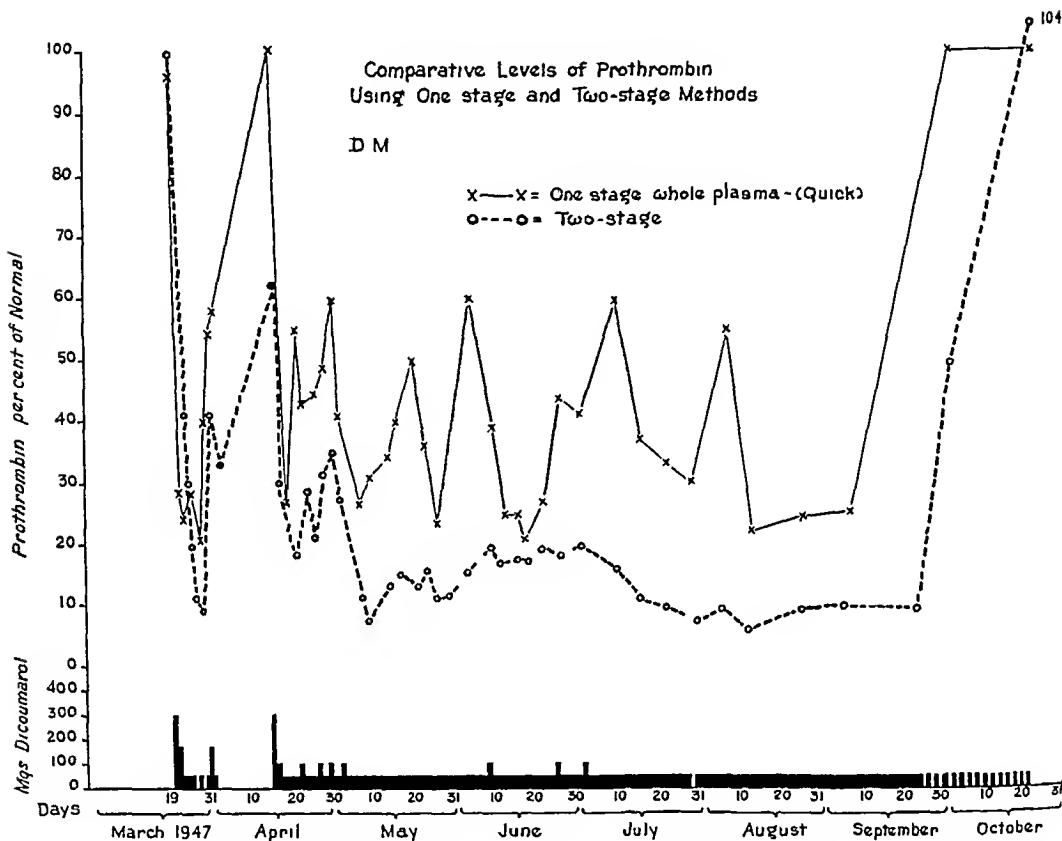


Fig 1—This chart represents the comparative prothrombin levels of a patient given Dicumaiol for a thrombophlebitis of the leg. Note the wide variations between the results of the two methods. According to the two-stage test the patient was well controlled on about 50 mg of Dicumaiol daily. Had the one-stage method been guiding the therapy he might at several points have been given 200 mg and the prothrombin depressed to bleeding levels.

In nearly all instances, the prothrombin estimate according to the unmodified two-stage method was 10 to 20 per cent lower than that of the modified two-stage procedure (Fig 3). This is consistent with the finding of Ware, Guest, and Seegers<sup>18</sup> that an increase in Ac-globulin is accompanied by an increase in the quantitative yield of thrombin from purified prothrombin. Variations between the two were not uniformly altered by marked variations in the Ac-globulin level. That is, when the Ac-globulin was low, the difference between the two tests did not uniformly become greater as might be expected (Fig 3).

The same situation was obtained with respect to the one-stage test with and without the serum factor. The range of variation between the two was

greater, however, than that between the two types of two stage tests, the widest difference being 52 per cent. This disparity did not uniformly follow variations in the A $\gamma$  globulin values (Fig 3)

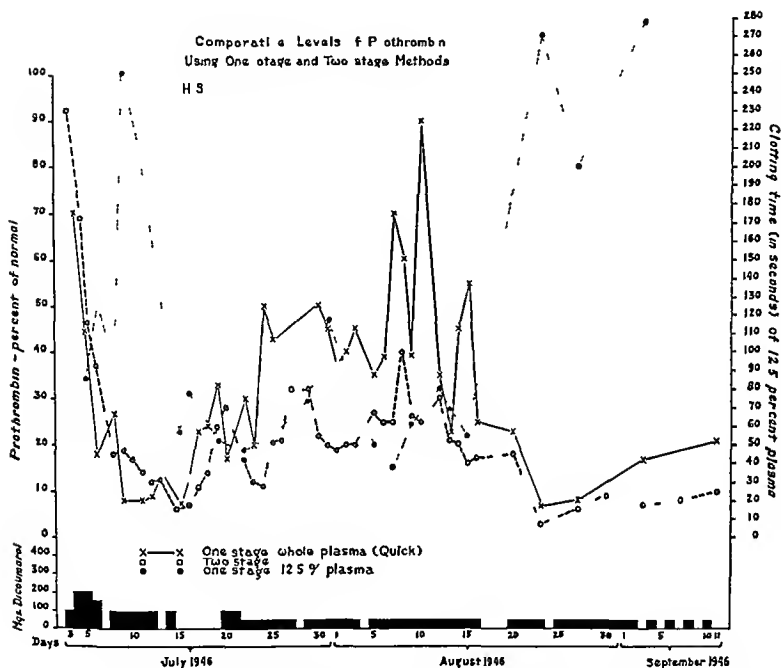


Fig. —This chart represents the comparative prothrombin levels of a patient given Dicumarol because of a coronary thrombosis. In addition to the two stage and the one stage whole plasma methods the results of the one stage 1.5 per cent plasma method are shown. The readings of the two former tests are given in percentage on the left hand ordinate. The results of the 1.5 per cent plasma method are given in seconds on the right hand ordinate. When the prothrombin is low the prothrombin time of the 12.5 per cent plasma would be expected to remain elevated. Neither of the one stage tests is uniform as compared with the results of the two stage method.

In patients on Dicumarol therapy the one stage method usually gives a figure higher than that of the two stage with or without the serum factor. In the first week of therapy however this relationship may be altered and the one stage give a lower figure than the two stage (Fig 1). In 85 per cent of the cases in which the unmodified tests were run in parallel, the one stage on one or more days in the first ten days of dicumarolization gave a lower figure than the two stage test. In 42 per cent the rate of conversion of prothrombin in the two-stage test was increased. In all others it was unchanged.

## COMMENT

Properly controlled Dicumarol therapy provides for the maintenance of a patient within a desired prothrombin bracket. In this laboratory the two stage method has made this possible, whereas the one-stage method on whole or diluted plasma by comparison has shown wide variations. It is believed

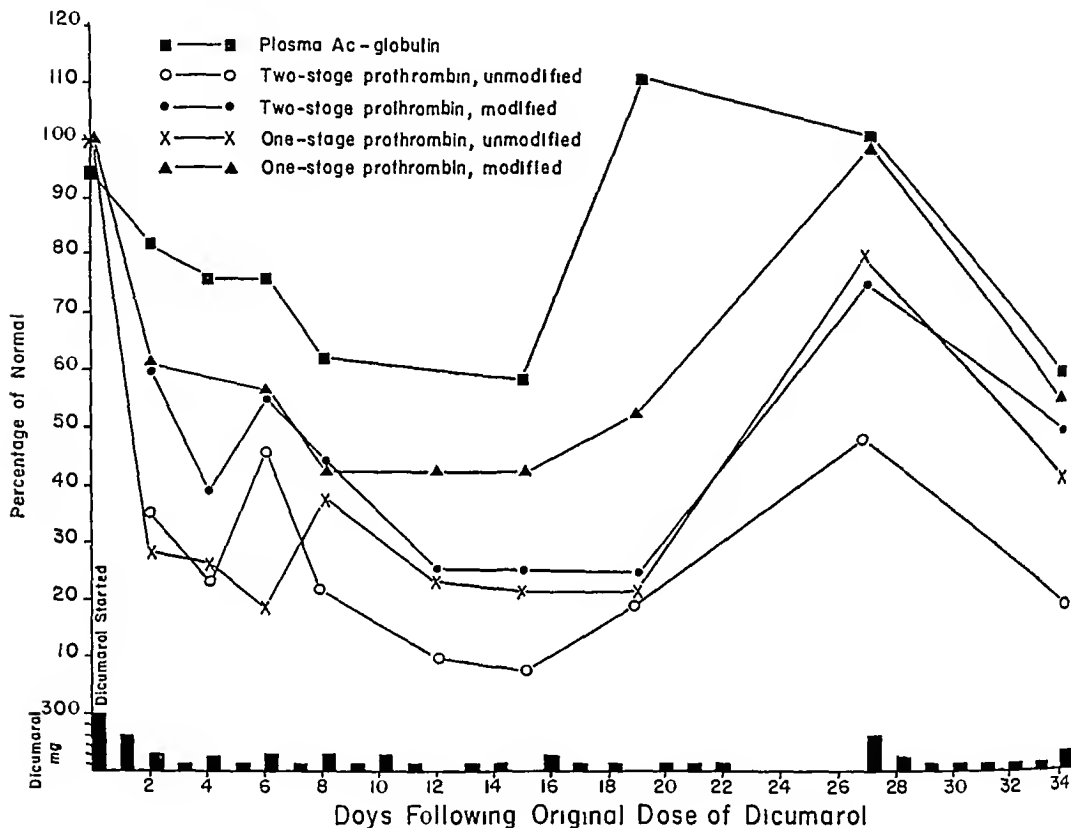


Fig 3—This chart shows the relative variations of the Ac-globulin levels as compared with the one stage and two stage prothrombin values during the early days of Dicumarol therapy. Note that the two-stage tests modified and unmodified parallel each other rather closely. The unmodified one-stage test early shows a level well below the two-stage level and later is well above the two-stage value. The Ac-globulin falls in the early days of therapy and has returned to normal by the end of nineteen days. Between the twenty-second and twenty-seventh days the patient failed to take dicumarol and the prothrombin returned toward its original level. When this again dropped toward therapeutic levels the Ac-globulin again dropped and the one-stage prothrombin levels again dropped more rapidly than did the two-stage values. There is no uniform correlation between the variations between the modified and unmodified prothrombin methods and the Ac-globulin values. The percentage values of the modified one stage test are based on the time percentage curve of the unmodified test.

that instances of fatal hemorrhage have occurred because of erroneous prothrombin estimates, and that variation in factors other than prothrombin often are responsible for the one-stage result. This is less often true of the two-stage method. The one-stage test is of value, however, as a summation of the various clotting factors. In instances where the two stage reading is below 10 per cent of normal, and the one stage result is above 10 per cent, bleeding is not likely. When both are below 10 per cent, bleeding may be im-



ninent As long as the prothrombin level is above 1 per cent according to the two stage test, the patient is never far below his bleeding threshold and bleeding may be controlled by proper measures

Fahey, Olwin, and Ware<sup>6</sup> found a drop in the A $\alpha$  globulin in the plasma of dogs during the first ten to fourteen days of dicumarolization This was followed by an elevation above the normal, and then a return to normal levels during subsequent therapy The same authors,<sup>6</sup> studying human patients found a drop in this factor during the early phases of dicumarolization and a return to normal after three weeks The reason for this fall in A $\alpha$  globulin during the early stages of dicumarolization is not clear If the factor is intimately associated with the prothrombin molecule, it might be expected to remain at a low level throughout Dicumarol therapy Regardless of extremely low prothrombin levels, however after the first three weeks in all cases studied the A $\alpha$  globulin remained within normal ranges Of practical consideration is the possibility that it might be a factor in hemorrhage if the prothrombin is early reduced to a low level

Hurn, Barker, and Mann<sup>10</sup> found that early in the dicumarolization of patients the one stage method always showed a lower figure than did the two stage, and suggested that this would seem to indicate a decreased rate of conversion of prothrombin to thrombin Owen and Bollman,<sup>11</sup> using dogs, found that when the one stage yielded lower results than the two stage, the conversion of prothrombin was delayed When the tests agreed, the conversion rate was normal, and when the one stage gave a higher result, conversion rates were accelerated above normal They suggested that the discrepancy between the one and two stage tests appears to reside in a conversion factor

The drop in A $\alpha$  globulin during the early stages of dicumarolization would support this premise The A $\alpha$  globulin remains depressed, however, longer than the one stage level remains below the two stage It might be expected that the addition of serum rich in A $\alpha$  globulin to human plasma would materially lessen the difference between the results of the one stage and two stage tests In this study such was not the case Furthermore, in this laboratory in most cases there was no change in the rate of conversion of prothrombin to thrombin when the one stage test gave a result lower than that of the two stage It is possible that other factors in the beef serum or human plasma may influence the activity of prothrombin It is also possible that Dicumarol may influence factors other than prothrombin and A $\alpha$  globulin The problem will require further study

#### SUMMARY

- 1 The two stage prothrombin determination provides for a more accurate and a safer control of prothrombin during Dicumarol therapy than does the one stage method

- 2 The one stage method is of value, however in estimating the summation of the various clotting factors A reading of below 10 per cent by the

two-stage test does not necessarily mean that hemorrhage is imminent, particularly if the one-stage gives a reading of above 10 per cent

3 In seven patients studied from the beginning of therapy, the Ac-globulin values fell during the early days of dicumarolization and returned toward normal by the end of twenty one days. In patients who had been on therapy over longer periods of time, some as long as eighteen months, there was no material variation from the normal

4 The addition of beef serum in small amounts to both the one- and two-stage tests raises the values obtained for prothrombin. Variations between the modified (with serum added) and unmodified (without serum added) methods do not follow fluctuations in the Ac-globulin values as obtained in this study. Neither do the fluctuations between the one stage and two-stage tests. These findings would suggest that other factors in addition to Ac-globulin are responsible for the disparity between the one-stage and two-stage methods

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# THE CAUSES FOR REJECTIONS OF BLOOD DONORS

## A STATISTICAL STUDY

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THE purpose of this paper is to present a statistical report of the causes for rejections of blood donors. Taylor and Heiss<sup>1, 2</sup> in previous papers have presented statistical reports covering the entire blood donor service of the American Red Cross.

It was felt that a more detailed report from a single large blood donor center might be of value at this time when blood procurement for civilian use is being undertaken.<sup>3, 4, 5, 6</sup>

The blood donor requirements used in this study were those set forth in the Technical Manual by the Technical Director of the American Red Cross Blood Donor Service.<sup>7</sup>

This report covers a seventeen-month period from January, 1944, through May, 1945, on donors who reported to the fixed center and the mobile unit of the American Red Cross Blood Donor Center of Chicago. Included in this report are causes for rejections whether permanent or temporary. During this period our percentage of repeat donors varied between 48 per cent and 62 per cent. The data presented no doubt include some duplications due to the fact that each time a donor registered a new number was assigned that donor.

A brief history and a preliminary examination was made on each donor prior to acceptance or rejection to rule out any acute infection or serious chronic illness.

A brief explanation of each donor requirement is presented.

A. Age. No donor was acceptable who was over 60 or under 18 years of age.

B. Minors. A minor was not acceptable unless he had a standard form signed by parent or legal guardian.

C. Underweight. The minimum weight for a donor was 110 pounds.

D. Anemia. This term was used to classify those donors who did not meet our hemoglobin requirement by our methods and our technique. In this category fell the highest number of rejections—49.84 per cent of all rejections. From January, 1944, until May, 1945, the Tallquist method for hemoglobin determination was used and a level of 80 per cent was necessary for acceptance. From May, 1945, on, the copper sulfate method<sup>8</sup> was used and it was necessary to have a level of approximately 12.3 Gm. to be acceptable.

E. Hypertension. The upper limit of the systolic pressure was 200 and the upper limit of diastolic was 110.

F. Hypotension. No donor was acceptable whose systolic pressure was below 100.

Data for this study were obtained from the American Red Cross Blood Donor Center Chicago, Ill.

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G Fatty foods Donors were refused or postponed who gave a history of having a fatty meal three to four hours prior to donation. This was done to avoid lipemia and there also was noted a tendency of reactions with gastrointestinal complications in donors who had eaten a heavy meal.

H Heart rate over 100 or under 60 (rejected) If any irregularity of pulse occurred the donor was checked by the physician in attendance and accepted or rejected according to his judgment.

I Temperature over 99.5 was not acceptable.

J Venepuncture in past eight weeks Donors were allowed to donate every eight weeks and were allowed five donations in a twelve month period.

K Cold in past week No donor with cold was acceptable to prevent false positive serologic reaction or transmission of disease in blood.

L Other recent illness or major surgery Donors were rejected who had had pneumonia yellow fever dengue or virus infections during the past six months. A donor was postponed until six months after major surgery. Minor surgery—judgment of the physician in attendance.

M Diabetes Anyone giving a history of diabetes was not acceptable even though the disease was controlled at that time.

N History of skull fractures or severe head injury with subsequent symptoms These donors were rejected; there seemed an increased tendency toward syncope in this group of donors.

O Psychosis and psychoneurosis

P Other chronic illness Malta fever marked arteriosclerosis history of liver disease chronic renal disease cerebral or coronary thrombosis were cause for rejection.

Q General physical debility General appearance of the donor as he appeared to the physician in attendance. Even though he met the minimum requirements the donor was rejected if deemed a "poor risk" by the physician in attendance.

R Pregnancy or childbirth No pregnant women were accepted or those who had delivered within the past nine months. No donor was accepted who had miscarried within the preceding six months.

S Malaria Any donor who had had malaria in the past fifteen years was not acceptable.

T Infectious or indeterminate jaundice or contact with one with jaundice Any donor who had jaundice within the past six months was disqualified. Each donor was asked whether any member of the immediate family had had jaundice within the past six months. A donor with contact was judged for acceptance only if it was obvious that the jaundice was not of the infectious type. A donor with jaundice in the immediate family was accepted only if there had been no contact. Taylor and Heiss<sup>2</sup> in their report found that in questioning three million donors 325 gave a history of jaundice in the past six months. Grossman and co-workers' present data which seem to indicate that a causative agent for hepatitis was being transmitted to patients through the medium of blood plasma and whole blood transfusion.

TABLE I

	MEN	WOMEN	TOTAL
Number of donors	158,858	216,910	375,768
Number rejected	15,865	63,876	79,741
Per cent of rejections	9.98	29.44	21.22

TABLE II

CODE	MEN			WOMEN			TOTAL
	NUMBER REJECTED	NUMERICAL SEQUENCE OF CAUSE OF REJECTIONS	PER CENT REJECTED	NUMBER REJECTED	NUMERICAL SEQUENCE OF CAUSE OF REJECTIONS	PER CENT REJECTED	
A Ages over 60, under 18	155	13	9.8	87	19	14	242
B Age 18-21 No written permission	141	14	8.9	623	11	9.8	764
C Underweight	496	10	3.13	4,486	2	7.02	4,982
D Anemia	1,892	2	11.93	38,005	1	59.50	39,897
E Hypertension	1,647	3	10.38	2,420	6	3.79	4,067
F Hypotension	477	11	3.01	3,698	4	5.79	4,175
G Fatty foods	94	18	5.9	193	16	3.0	287
H Pulse rate over 100 or under 60	1,460	5	9.20	3,944	3	6.17	5,404
I Temperature over 99.5	497	9	3.13	2,184	7	3.42	2,681
J Venepuncture in past 8 weeks	115	15	7.2	59	22	0.9	174
K Cold in past week	4,197	1	26.46	3,073	5	4.81	7,270
L Other recent acute illnesses or major surgery	1,031	6	6.50	988	9	1.55	2,019
M History of diabetes	64	20	4.0	51	23	0.8	115
N History of skull fracture or severe head injury and subsequent symptoms	47	22	3.0	28	24	0.4	75
O Psychoses and Psycho neurosis	48	21	3.0	64	21	1.0	112
P Other chronic illnesses	1,511	4	9.52	1,261	8	1.97	2,772
Q General physical debility	104	16	6.6	200	15	3.1	304
R Pregnancy or childbirth within last 9 mo				395	13	6.2	395
S Malaria	257	12	1.62	127	17	2.0	384
T Infectious and indeterminate jaundice or contact with jaundice	10	24	0.6	10	26	0.2	20
U History of frequent vertigo, syncope, convulsions	73	19	4.6	387	14	6.1	460
V Donor refused venepuncture	10	24	0.6	18	25	0.3	28
W Valvular heart disease	590	8	3.71	552	12	8.6	1,142
X History of anemia	22	23	1.4	103	18	1.6	125
Y History of tuberculosis	98	17	6.2	82	20	1.3	180
Z Other causes for rejections miscellaneous	829	7	5.23	838	10	1.31	1,667

U History of frequent attacks of vertigo, syncope, or convulsions was cause for rejection

V Donor refused venepuncture Occasional donors would change their minds about donation after they entered the bleeding room

W Any donor who gave history of valvular heart disease was checked by the physician in charge Each donor was questioned about shortness of breath, swelling of feet or ankles persistent cough and pain in chest, final decision was made by physician in charge

X History of anemia If at all suspicious, donor was rejected

Y History of pulmonary tuberculosis (rejected) Questions regarding extrapulmonary tuberculosis were referred to the physician in charge and each case was decided on the extent of lesion and time elapsed since its occurrence

Z Other causes for rejections

(a) Rabies treatment within last five years

(b) Chronic sinusitis or hay fever in acute stages

(c) Donors who had had extensive or prolonged sulfa therapy

(d) Severe or septic sore throat in preceding three months

(e) Vaccinations for smallpox or other immunizations recently Smallpox may produce false positive serology

(f) Army flight personnel were not acceptable donors

(g) Filariasis

In Table I is presented the total number of donors registered and rejected

In Table II are presented the causes for the preceding rejections

#### SUMMARY

1 In our report covering a period of seventeen months of operation at the Chicago Blood Donor Center 375 768 donors reported to the fixed center and mobile units

2 This group was represented by 158 858 men (42.27 per cent) and 216,910 women (57.73 per cent)

3 The rejection or postponement rate was 9.98 per cent in men and 29.44 per cent in women

4 The greatest causes for rejections for any single condition were recent colds in men and anemia in women

5 It is hoped that this study will be of value in the future procurement of blood donors

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## DISTRIBUTION OF EMETINE IN TISSUES

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THE alkaloid emetine is obtained from ipecac or prepared synthetically by methylation of cephaelline. Ipecac has been used in the therapy of diarrheas for more than 300 years and its alkaloids were investigated more than 100 years ago. The specific usefulness of ipecac in amebic dysentery was disclosed only after the discovery in 1875 that *Endamoeba histolytica* was the causative organism of this disease. Vedder<sup>1</sup> in 1912 demonstrated that high dilutions of emetine were capable of killing *E. histolytica*. It remained for Rogers<sup>2</sup> soon thereafter to prove the value of parenteral emetine injections in the treatment of amebiasis.

In 1920 Matter<sup>3</sup> in France reported, without disclosing his method of analysis, that emetine appeared in the urine within twenty minutes after injection and the excretion lasted for from five to nine weeks. Despite the fact that there are numerous papers dealing with the pharmacology and toxicity of emetine, no papers dealing with distribution of the alkaloid in animal tissues are reported. It is the purpose of this paper to present the data on the distribution of emetine in tissues of rabbits.

### EXPERIMENTAL

**Method**—Each of fifty-seven albino rabbits weighing about 2.5 kilograms was injected intramuscularly with 6 mg. per kilogram of emetine hydrochloride in aqueous solution (Higher doses frequently resulted in the death of the animals). The animals were sacrificed, in groups of three at intervals varying from one-half hour to sixty-four days after injection, as shown in Table I. The following organs were analyzed for emetine: brain, leg muscle, heart, mesenteric lymph node, liver, spleen, kidney, lung, and a portion of large intestine freed of feces. The organs of six noninjected rabbits were analyzed to determine the range of the blank determination. Those results which fell within the blank range were considered as zero.

**Determination of Emetine**—The method used in this study was adapted with some modifications from the general method of Biedie and co-workers<sup>4</sup> for the determination of organic bases in biologic material by salt formation with methyl orange. It depends upon the extraction of emetine from an alkaline medium by benzene, combining the base with methyl orange, extracting with acid the methyl orange salt formed, and measuring photometrically the amount of color due to the salt.

**Reagents**—1. Standard solution of emetine—20 mg. per 100 cubic centimeters. Twenty milligrams of emetine hydrochloride are dissolved in 100 cc. 0.01N HCl. This solution is stored in a refrigerator and keeps for weeks.

2. Metaphosphoric acid 6.5 per cent.

3. Saturated solution of sodium hydroxide.

4. Benzene. A reagent grade is redistilled.

5. Isoamyl alcohol. A reagent grade is redistilled.

6. Methyl orange solution. Prepared according to Biedie and associates.<sup>4</sup>

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TABLE I RECOVERY OF EMETINE AFTER ADDITION OF 5  $\mu$ G TO 0.2 GM. OF TISSUE

TISSUE	EMETINE FOUND ( $\mu$ O)	RECOVERY (%)
Brain	50	100
Lung	49	98
Lymph node	40	80
Spleen	51	102
Liver	56	112
Heart	53	106
Large intestine	53	106
Kidney	51	102
Muscle	48	96

*Procedure*—Take a known weight of tissue and grind with 20 vol of 6% per cent metaphosphoric acid in a Waring Blender. Grind soft tissue (brain, lung, etc.) forty five seconds and other tissues (liver, kidney, etc.) for one minute. Filter and use 3 to 6 cc of filtrate for the determination. Add 3 to 6 cc of filtrate (containing up to 20  $\mu$ g of emetine) to a 30 cc glass stoppered cylindrical separatory funnel. Add 1 cc of saturated NaOH and shake to neutralize the metaphosphoric acid and to have an alkaline solution for the extraction. Add 15 cc of benzene and shake for five minutes on a shaking apparatus then allow the phases to separate by standing forty five minutes to one hour. Add 0.25 cc. of isoamyl alcohol and mix well with only the benzene phase, taking great care not to disturb the aqueous phase. Draw off the latter and transfer all of the benzene phase to a screw capped test tube to which has been added 0.25 cc of methyl orange. Cap and shake in a shaking apparatus for five minutes and then centrifuge at 1000 rpm for ten minutes. Transfer 10 cc of the benzene phase to a glass stoppered centrifuge tube to which have been added 3 cc of 1N HCl. Shake five minutes and then centrifuge for five minutes at 1,000 rpm. Carefully remove the benzene phase by aspiration. Read the acid phase in a Beckman spectrophotometer at 510 millimicrons.

*Recovery*—To each 0.2 Gm. of various tissues from two untreated rabbits were added 5  $\mu$ g of emetine. Six cubic centimeters of tissue filtrate containing 5  $\mu$ g of emetine were then analyzed in duplicate according to the described procedure. Table I shows the amount of emetine recovered. These figures indicate that the drug can be recovered quantitatively from all tissues except possibly lymph node where only 80 per cent was recovered.

The method was used to determine as low as 0.2  $\mu$ g in 6 ml of filtrate, this being equivalent to 10 mg of emetine per kilogram of tissue. Figures below that amount were found to be within the variation of the blanks.

#### RESULTS

The tissue concentration of emetine at the various time intervals after injection are shown in Table II. Within one half hour the highest concentrations of drug were found in the lung, kidney, and spleen. The liver and heart contained much less, whereas the intestinal level was relatively very low. Emetine was found in very low amounts in striated muscle of the leg up to two hours after injection. Beyond this time the drug was not detectable. Questionable traces of emetine were found in the brain but most of these were within the limits of the blank and were recorded as negative.

In most organs the maximum concentration of drug occurred within twelve to twenty four hours after injection. This level persisted with slight change through the second day and then gradually began to decline. By the fourth day the concentration in the major organs was about half what it was at the maximum. After two weeks some of the organs were free of emetine, but the

spleen, lymph node, and kidney contained significant quantities of drug for as long as four weeks. Between six and nine weeks, the spleen, the last organ to contain emetine, became free of the drug.

TABLE II TISSUE CONCENTRATION OF EMETINE (MG/KG) AFTER AN INTRAMUSCULAR INJECTION OF 6 MG/KG, CONCENTRATIONS ARE AVERAGES OF THREE RABBITS STUDIED AT EACH TIME INTERVAL

TIME INTERVAL	MUSCLE	BRAIN	HEART	LUNG	LIVER	SPLEEN	KIDNEY	LARGE INTES TINE	LYMPH NODE
1/2 hr	0	0	12.4	46.7	12.6	18.7	39.1	3.3	2.0
1 hr	1.0	0	17.2	60.9	24.9	23.9	60.3	5.5	6.0
2 hr	1.0	0	10.7	64.1	28.3	67.9	60.3	3.2	4.0
6 hr	0	0	3.5	42.6	31.4	70.3	73.1	2.1	5.7
12 hr	0	0	3.5	58.8	41.6	70.3	101.4	1.7	6.9
16 hr	0	0	4.2	37.0	41.2	63.5	68.7	2.1	8.6
1 day	0	0	4.5	48.8	40.5	66.0	59.9	1.7	9.2
2 days	0	0	3.9	38.2	31.5	56.6	65.7	2.2	12.3
3 days	0	0	2.6	30.3	19.2	45.2	37.1	1.7	10.7
4 days	0	0	1.8	29.9	15.4	44.8	45.6	1.2	9.2
6 days	0	0	2.3	15.9	10.3	36.4	46.7	0	9.4
10 days	0	0	1.1	7.5	4.9	14.7	22.5	0	6.1
13 days	0	0	0	3.2	2.7	11.5	14.1	0	3.8
20 days	0	0	0	0	2.1	8.6	4.4	0	2.7
24 days	0	0	0	0	1.0	3.3	4.8	0	1.9
28 days	0	0	0	0	1.0	2.2	1.6	0	2.0
35 days	0	0	0	0	0	2.2	0	0	0
42 days	0	0	0	0	0	1.4	0	0	0
64 days	0	0	0	0	0	0	0	0	0

#### DISCUSSION

In a preliminary report<sup>4</sup> it was suggested that the difference in efficacy of emetine in intestinal and hepatic amebiasis was due to the difference in concentration of drug in the intestine and liver. A high, sustained level in the liver probably accounts for a high percentage of cures in hepatic disease, whereas the low, transient level in the large intestine could account for the frequent relapses of dysentery in cases where emetine alone is used. With similar reasoning, it appears that amebic brain abscess would not respond to parenterally administered emetine because the drug does not enter the brain in significant amounts.

The most disastrous effects of emetine in man are due to its cardiac effects. Soon after injection in rabbits, the heart muscle level of the drug is moderately high as compared with other organs. By six hours this drops to a relatively low level at which it remains until disappearance about ten days later. In man where the drug is given in divided daily doses, electrocardiographic changes after emetine therapy usually occur several days after treatment has begun.<sup>6</sup> These repeated doses are presumably additive insofar as cardiac concentration of drug is concerned. By the fifth or sixth day there is sufficient drug and also sufficient time elapsed for the drug to act on the muscle and electrocardiographic changes become evident. It would not be safe to repeat a course of therapy with emetine until complete excretion of the drug from the heart had occurred and also after a lapse of sufficient time for the damage to the heart to be repaired. From human studies, this is probably about two months.<sup>6</sup>

Very little is known about the mechanism of action of emetine in amebiasis. It is considered a general protoplasmic poison. However, in high dilutions it is

only effective against the motile trophozoite form of *E. histolytica* and not against the encysted form. In the present experiments it was shown that the highest concentrations of drug were found in the liver, spleen, kidney, and lung, although in man few or no toxic signs and symptoms from emetine can be related to these organs as shown by Klatskin and Friedman.<sup>8</sup> In fact, they noted that some patients with hepatic amebiasis had abnormal liver function studies and that these abnormalities actually returned to normal under the influence of emetine. The lowest concentrations of drug in the rabbits were found in cardiac muscle, striated muscle, and intestinal muscle. Yet, despite the low concentration of drug in the heart, muscles and intestine, almost all toxic symptoms in man<sup>8</sup> and animals<sup>9</sup> are associated with these organs. In other words, only those organs having contractility as their most important function are affected adversely even though the concentration of drug in these organs is not inordinately high. On this basis, it is postulated that emetine interferes in some way with the chemical function of the cell (perhaps an enzyme system), which converts glycogen into contractile energy.

#### SUMMARY

The tissue distribution of emetine in rabbits after a single injection has been determined. The drug is found in highest concentration in the lung, kidney, spleen, and liver. The concentration in the heart and lymph nodes is intermediate and that in the large intestine and striated muscle is very low. The drug persists for as long as four to six weeks in some organs. Significant amounts of the drug were not detectable in the brain, indicating that parenteral emetine would probably be ineffective therapy in amebic brain abscess.

On the basis of the finding that emetine is most toxic for the various muscular organs despite the low concentration of drug in these organs, it is postulated that emetine interferes with a biochemical function of those cells having contractility as their main function.

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## A SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF PROCAINE AND *p*-AMINO BENZOIC ACID

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SINCE procaine is used widely in clinical medicine, a good method for the quantitative estimation of this drug is desirable. Acidimetric,<sup>1-5</sup> iodometric,<sup>6</sup> mercurimetric,<sup>7</sup> bromometric,<sup>8-13</sup> and gravimetric<sup>14-16</sup> methods have been proposed in the past. Vanillin,<sup>16-17</sup> sodium cuprihydrosulfite,<sup>18-19</sup> thymol,<sup>20</sup> and pyridine<sup>20</sup> have been used for the detection of procaine. It also has been analyzed by the use of its absorption spectrum.<sup>21</sup> A spectrophotometric method of determination of procaine in procaine penicillin G at the wavelength of 290 mμ was recently reported.<sup>22</sup> After the present work was completed, a spectrophotometric method for procaine and its hydrolytic products was described by Biedie and co-workers.<sup>23</sup>

The most useful methods, however, depend on the formation of a colored compound after procaine is diazotized.<sup>24-28</sup> In 1939 Bratton and Marshall<sup>29</sup> reported that *N*-(1-naphthyl) ethylene diamine dihydrochloride was the most suitable coupling reagent for sulfanilamide determinations. Since then this method has been adopted extensively for the estimation of local anesthetics of the *p*-aminobenzoic acid ester type.<sup>30-32</sup>

Because procaine and one of its decomposition products, *p*-aminobenzoic acid, give rise to similar colors in this coupling reaction, it is necessary to differentiate these two compounds in the presence of one another. Ether,<sup>24-28, 31, 32</sup> chloroform,<sup>33</sup> and isopropyl alcohol<sup>36</sup> have been used to separate the two. These procedures required repeated extractions and separations, later followed by the development of the color for procaine and for *p*-aminobenzoic acid separately. Hence the procedures were very complicated.

Legge and Durie<sup>37</sup> separated the procaine and *p*-aminobenzoic acid after the color was formed by means of hydrochloric acid. However, these workers did not describe the sensitivity and accuracy of their method. Hazard and Ravasse<sup>38</sup> separated the two azo dyes by means of ether in the presence of alkali, but they did not measure quantitatively the procaine itself.

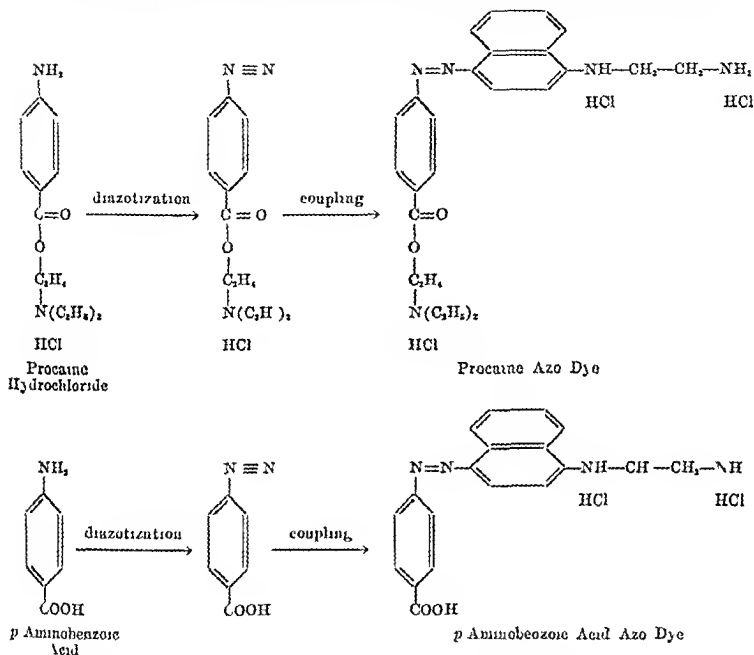
The present study has involved the development of a modification of the foregoing methods in order to have an accurate and sensitive procedure for the determination of procaine and *p*-aminobenzoic acid in the presence of each other in blood. The procedure is simpler than any of the methods previously described.

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## EXPERIMENTAL

*Principle*—Blood protein is precipitated by trichloroacetic acid. The supernatant clear fluid is diazotized. Ammonium sulfamate is used to destroy the excess nitrous acid. The color is developed by coupling with N (1 naphthyl) ethylene diamine dihydrochloride.



Then the solution is treated with chloroform and alkali. The color intensity is measured in the Coleman Model 6A Junior spectrophotometer, using adapter #6-108 and cuvette #6-310B.

The orange-red color remaining in the aqueous phase represents the p-aminobenzoic acid azo dye, which shows maximum absorption at the wave length of 500 mμ using water for zero setting. The yellowish orange color in the chloroform represents the procaine azo dye, which shows maximum absorption at the wave length of 485 mμ using chloroform for zero setting. The optical density curves of these two products are shown as curve 1 and curve 2 in Fig. 1.

*Reagents*—

- 1% per cent trichloroacetic acid
- 0.1 per cent sodium citrate
- 0.5 per cent ammonium sulfamate
- 0.1 per cent N (1 naphthyl) ethylene diamine dihydrochloride
- Chloroform
- 1N sodium hydroxide

*Procedure*—Pipette 0.1 ml of oxalated blood into 30 ml of water, and let stand for three minutes. Add 1.0 ml of 1% per cent trichloroacetic acid and let stand for five minutes. Centrifuge at 1500 rpm for five minutes. Pipette 3.0 ml of the supernatant

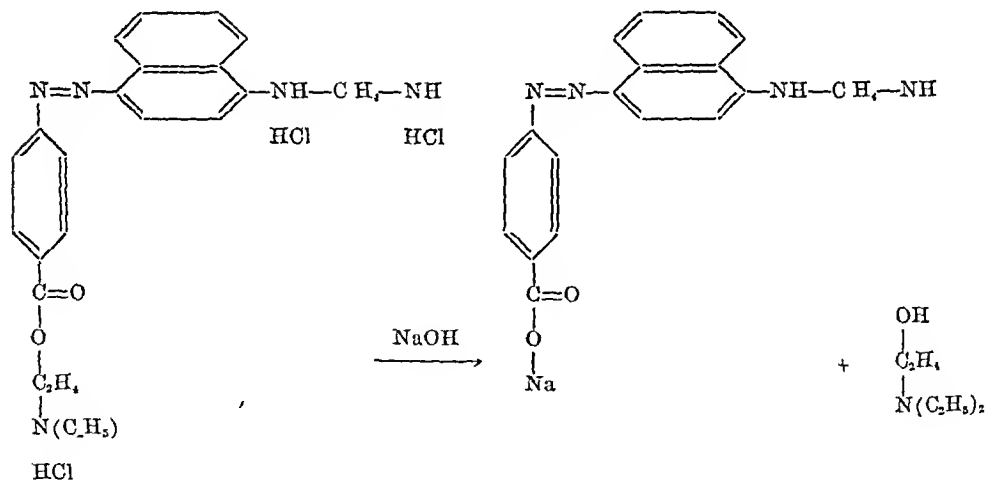
clear solution into a Maizel Gerson reaction tube. Add 0.2 ml of 0.1 per cent sodium nitrite, and let stand for three minutes. Add 0.2 ml of 0.5 per cent ammonium sulfamate, and let stand for two minutes. Add 0.2 ml of 0.1 per cent *N*-(1-naphthyl) ethylene diamine dihydrochloride, and let stand for ten minutes. Add 3.0 ml of chloroform, and shake for half a minute. Then add 0.8 ml of 12*N* sodium hydroxide. Shake vigorously for two minutes. The solution should be well mixed after each addition of reagent.

Pipette out about 2 ml of the aqueous solution for spectrophotometric determination of *p*-aminobenzoic acid at the wave length of 500  $m\mu$  using water for zero setting. Pipette out about 2 ml of the chloroform solution for spectrophotometric determination of procaine at the wave length of 485  $m\mu$  using chloroform for zero setting. Reagent blanks should be used in each series of determinations.

**Effect of Order of Reagents**—The shaking with chloroform before the addition of alkali is important. If the chloroform is added after the alkali, the extraction of procaine azo dye will be incomplete and a part of the dye will remain in the aqueous solution. For instance, a sample containing 30  $\mu$ g of procaine hydrochloride and 30  $\mu$ g of *p*-aminobenzoic acid was analyzed according to the procedure except that the sodium hydroxide was added ten minutes after *N*-(1-naphthyl) ethylene diamine dihydrochloride, then chloroform was added. The spectrophotometric reading of the chloroform solution gave an optical density of 0.271, which corresponded to about 21  $\mu$ g of procaine hydrochloride. Therefore the extraction was only two thirds complete. The spectrophotometric reading of the aqueous solution gave an optical density of 0.537, corresponding to about 36  $\mu$ g of *p*-aminobenzoic acid.

This phenomenon may be explained by means of polarity. When the procaine azo dye solution is first shaken with chloroform, the ester portion of the dye goes into

the chloroform phase while the  $-N$  HCl part remains in the aqueous phase. The subsequent addition of alkali neutralizes the HCl, freeing the amino group. Hence the whole molecule of the procaine azo dye goes into the chloroform. On the other hand, if the alkali is first added prior to the chloroform, the ester group may be hydrolyzed in the following manner:



This sodium salt thus produced is less soluble in chloroform.

**Effect of Amount of Alkali**—The amount of alkali added is important in the determination of *p*-aminobenzoic acid. The sodium hydroxide solution was tested in volumes of 0.2, 0.4, 0.5, 0.7, 0.8, and 1.0 milliliter. It was found that 0.8 ml of sodium hydroxide gave the best result. The amount of alkali is, however, not so important in the determination of procaine. The same results were obtained using 0.2 ml and 0.8 ml of sodium hydroxide.

**Stability of Color**—The color of the procaine azo dye is remarkably stable. There was no significant fading of the color even after the chloroform solution was stored in the refrigerator for eight weeks. The color of the *p*-aminobenzoic acid azo dye is much less stable. About 25 per cent of the color intensity of the latter dye will fade in four hours, and this color will bleach out almost completely after one day. For this reason, the measurement of the *p*-aminobenzoic acid should be carried out as quickly as possible.

**Prevention of *in Vitro* Hydrolysis of Procaine in Blood**—Since blood contains procaine esterase, highest in the human being,<sup>33</sup> the procaine disappears rapidly from a drawn

sample of oxalated blood. When sodium fluoride (about 4 mg per milliliter) was added to the blood sample or if the blood sample was kept in ice the hydrolysis was greatly retarded. When combined fluoride and low temperature were used immediately after the blood was drawn, the procaine hydrolysis was completely inhibited, as shown in Table I.

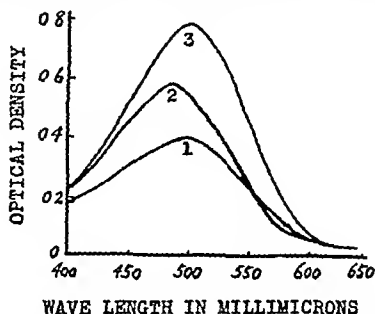


Fig 1—Spectrophotometric absorption curves. Curve 1 *p* aminobenzoic acid azo dye in aqueous phase; curve 2 procaine azo dye in chloroform; curve 3 sulfanilamide azo dye in aqueous phase.

TABLE I. HYDROLYSIS OF PROCAINE ADDED TO FRESH HUMAN BLOOD

	μG PROCAINE AT ZERO TIME	μG PROCAINE AFTER 1 HR	PER CENT LOSS
Oxalated blood (25 C)	37.0	13.5	64
Oxalated blood (0.5 C)	69.0	65.4	5
Oxalated blood + NaF (25 C)	50.0	48.0	4
Oxalated blood + NaF (0.5 C)	42.5	42.5	0

**Results of Analysis**—Different amounts of procaine hydrochloride were added to 0.2 ml of fresh oxalated dog or human blood to which sodium fluoride was added 4 mg per milliliter, and the tubes were kept in ice. Analysis for procaine was done according to the foregoing procedure and the results were obtained as illustrated by line 1 in Fig 2. When different amounts of *p* aminobenzoic acid were added to the blood and analyzed, the plotted results gave line B in Fig 2. When the blood was added to different amounts of procaine hydrochloride containing 50 μg of *p* aminobenzoic acid in each sample, the results of the analysis for procaine still gave line 1. When the blood was added to different amounts of *p* aminobenzoic acid containing 50 μg of procaine hydrochloride in each sample and analyzed the results of the analysis for *p* aminobenzoic acid still gave line B. The deviation of individual determinations from the line for procaine was usually less than that for *p* aminobenzoic acid.

Six 0.2 ml samples of human blood each containing 50 μg of procaine hydrochloride and various amounts of *p* aminobenzoic acid in each were analyzed. The spectrophotometric readings of the chloroform solution for the determination of procaine are shown in Table II.

TABLE II DETERMINATION OF PROCAINE IN THE PRESENCE OF *p* AMINO BENZOIC ACID

PROCAINE HCl (μg)	<i>p</i> AMINO BENZOIC ACID (μg)	OPTICAL DENSITY
50	0	0.63
50	5	0.64
50	10	0.62
50	20	0.61
50	30	0.62
50	50	0.64
		Mean 0.63

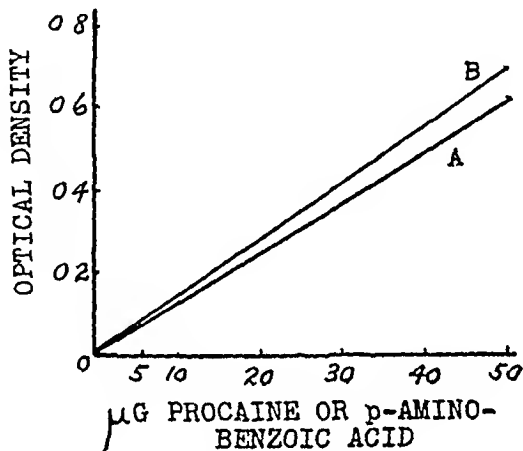


Fig. 2—Results of determination of procaine and *p* aminobenzoic acid under conditions described in text

Six 0.2 ml samples of human blood each containing 50 μg of *p*-aminobenzoic acid and various amounts of procaine hydrochloride were analyzed. The spectrophotometric readings of the aqueous solution for the determination of *p* aminobenzoic acid are shown in Table III.

TABLE III DETERMINATION OF *p* AMINO BENZOIC ACID IN THE PRESENCE OF PROCAINE

<i>p</i> AMINO BENZOIC ACID (μg)	PROCAINE HCl (μg)	OPTICAL DENSITY
50	0	0.71
50	5	0.70
50	10	0.69
50	25	0.71
50	50	0.70
50	50	0.71
		Mean 0.70

*Determination of Sulfanilamide*—According to this method, sulfanilamide formed an azo dye which remained in the aqueous solution and gave a maximum absorption at the wave length of 500 mμ as shown by curve 3 in Fig. 1. Sulfanilamide, in the absence of *p*-aminobenzoic acid, could be determined by this method in a manner similar to that employed for *p*-aminobenzoic acid. Since



hydrolysis of sulfanilamide does not occur in blood, sodium fluoride and low temperature are not necessary. The result of analysis was quite similar to that of *p* aminobenzoic acid.

#### DISCUSSION

Blood samples containing less than 25  $\mu\text{g}$  of procaine hydrochloride *p* aminobenzoic acid, or sulfanilamide in 0.2 ml will give a reading below optical density 0.05, which represents more than 10 per cent effective error.

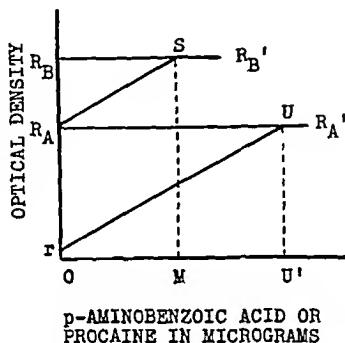


Fig 3—Diagram of calculation by internal standard

of the instrument used (Coleman Model 6A Junior spectrophotometer). Samples containing more than 5 to 7  $\mu\text{g}$  will give an optical density above 0.10, which represents less than 5 per cent instrument error. The effective instrument error in the range of optical density between 0.30 and 0.70 is below 2 per cent.

A reagent blank might be used for zero setting. However, this was less constant than when water or chloroform was used. The reagent blank usually gave an optical density below 0.010. If the optical density becomes too high (e.g., the coupling reagent solution turns to a pinkish color) the reagents should be changed.

Citrate was tested as an anticoagulant instead of oxalate and was found to give similar results. The coupling reagent *N* (1 naphthyl) ethylene diamine dihydrochloride, should be stored in the refrigerator and must be colorless when used.

Sulfanilamide as predicted from the closer similarity of its chemical structure to *p* aminobenzoic acid than to procaine interferes with the determination of *p* aminobenzoic acid but not with that of procaine.

Blood samples of the mouse, rat, dog, and man were analyzed with satisfactory results. This method is also applicable to any kind of biological materials. A specific reference line should be prepared in each case.

For each series of determinations, a reference line should be made from the blank and from the blank with a known amount of standard added. If a reference line is not available, a simple calculation of results based on the principle of "internal standard" may be used. This can be done in the following manner. Divide the sample into two equal parts, part A and part B. To part B add a suitable amount,  $M$ , (Fig 3) of procaine hydrochloride and *p*-aminobenzoic acid. Then perform the analysis of these two parts separately according to the method outlined. Let the spectrophotometric readings be  $R_A$  and  $R_B$  for part A and part B respectively. Locate the  $R_A$  and  $R_B$  on the ordinate, and draw two horizontal lines as  $R_A R_A'$  and  $R_B R_B'$ . Locate  $M$  on the abscissa and draw a vertical line which intersects  $R_B R_B'$  at  $S$ . Now draw a sloping line passing through the reagent blank reading 1 and parallel to  $R_A S$ . This line intersects  $R_A R_A'$  at  $U$ . The abscissa of the point  $U$ , or  $OU'$ , represents the amount of procaine hydrochloride or *p*-aminobenzoic acid present in the unknown sample part A.

#### SUMMARY

A simple spectrophotometric method for determination of procaine and *p*-aminobenzoic acid in blood in the presence of one another is presented. The sensitivity is 25 micrograms.

Fluoride and low temperature should be used *in vitro* to inhibit hydrolysis of procaine in the blood sample.

Sulfanilamide, in the absence of *p*-aminobenzoic acid, also can be determined by this method. Sulfanilamide interferes with the determination of *p*-aminobenzoic acid but not with that of procaine.

A simple method of calculation by means of "internal standard" is described.

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# THE KEPLER WATER TEST IN TABES DORSALIS

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N 1941 Robinson, Power, and Kepler<sup>1</sup> devised the water test as an aid in the diagnosis of Addison's disease. Soon other unrelated conditions were found to result in a positive test. According to Kepler, Power, and Levy,<sup>2</sup> these included chronic nervous exhaustion, cachexia, hyperthyroidism, liver disease, pituitary insufficiency, and renal disease.

The present report deals with nine patients having tabes dorsalis who were observed to give a positive result to the water test. Some of these had symptoms suggestive of adrenal hypofunction. None had any of the conditions previously reported as responsible for a positive test. Seven of these patients had a positive Kahn reaction in the spinal fluid and blood.

Table I summarizes the clinical and laboratory data, including the results of the water test on these nine patients with syphilis of the central nervous system. Some of these patients had been treated for neurosyphilis with penicillin and fever therapy. Neurological findings were minimal in a few instances (Patients 1, 7, and 8). All the nine patients were found to have orthostatic hypotension. The urine analyses done prior to the tests were normal in all the nine cases. Cystometric studies were performed in each patient to eliminate the possibility of a neurogenic bladder. Patient 8 with a positive Wilder test did have an early neurogenic bladder, but no residual urine could be found by catheterization. The state of hydration in all the patients was satisfactory by clinical observations, i.e., normal turgor of the skin, normal blood pressure, and normal daily urine output.

During the performance of this test the patients remained in bed and were observed carefully to insure proper ingestion of the calculated volume of water. The last urine specimen was collected by catheterization.

## RESULTS

The results show that five patients had a positive value for factor A, two were borderline, and one was negative. The volumetric part of the test was positive in all patients. There was a slight increase of the blood urea nitrogen values in five instances. Only Patients 5 and 6 had plasma chloride ion values below normal levels.

Table II shows a comparison of the individual items in the water test between the normal individual patients with Addison's disease (average values in twenty-four untreated cases)<sup>3</sup> and patients with tabes dorsalis (average in the nine cases). It is clear that the variation from normal tests was due to an abnormal volumetric part (delayed water diuresis). The average values of the plasma and urine chloride ion and urine urea nitrogen were within normal limits. As previously mentioned, there was a slight increase in the blood urea nitrogen in five out of the nine patients.

TABLE I. DETAILS OF THE KIDNEY WATER TEST IN PATIENTS WITH TABES DORSALIS

PATIENT UNIVERSITY HOSPITAL NO.	AGE	BLOOD PRESSURE	101 UNMETRIC PART					CHEMICAL PART				
			NIGHT SAMPLE (CC)	9 30 (CC)	10 30 (CC)	11 30 (CC)	12 30 (CC)	PLASMA CHLORIDE ION (MG %)	BLOOD UREA (MG %)	UPINE CHLORIDE (MG %)	UPINE UREA (MG %)	FACTOR A
1 W C	30	L <sub>2</sub> 130/70 Std 70/50	320	105	110	75	60	320	13.4	32.4	3,205	7.73
533239												
2 F K	47	L <sub>2</sub> 150/85 Std 90/60	450	130	180	140	110	321	27.5	126	1,606	60.0
307301												
3 F K	46	L <sub>2</sub> 120/80 Std 100/65	300	100	90	200	200	304	21.5	3.3	1,050	20.5
005041												
4 J W	54	L <sub>2</sub> 200/100 Std 140/80	200	50	100	90	00	374	20.0	46.4	1,500	24.3
600176												
5 H C	50	L <sub>2</sub> 120/70 Std 80/40	320	23	20	20	50	303	14.2	60	1,626	9.2
589615												
6 C V	64	L <sub>2</sub> 110/80 Std 80/40	375	50	105	185	165	312	4.6	306	1,662	21.8
585513												
7 L K	43	L <sub>2</sub> 90/60 Std 80/50	400	60	105	120	120					
590210												
8 C F	44	L <sub>2</sub> 100/60 Std 85/60	450	250	255	140	130	330	22.6	624	1,746	22.0
561918												
9 C S	54	L <sub>2</sub> 140/80 Std 90/70	500	30	210	285	120	335	13.7	120	1,487	25.2
599890												

L<sub>2</sub> indicates the blood pressure when lying and Std the blood pressure when standing

TABLE II THE ROBINSON, POWER, KEPLER WATER TEST IN NORMAL SUBJECTS, IN PATIENTS WITH ADRENAL INSUFFICIENCY, AND IN PATIENTS WITH TABES DORSALIS

ITEM	NORMAL PERSON (GENERAL DIET)	ADRENAL INSUFFICIENCY (UNTREATED)* (GENERAL DIET) <sup>3</sup>	TABES DORSALIS AVERAGE VALUES FROM 9 PATIENTS (GENERAL DIET)
Night urine volume (nine hour period) (c c)	200	300 400	407
Largest fractional urine specimen (c c per hour)	400 600	0 60 100	171
Plasma chloride ion (mg %)	320 350	228	332
Blood urea nitrogen (mg %)	10 15	39	20
Urine chloride ion (mg %)	250 350	560	306
Urine urea nitrogen (mg %)	1,600	1,000 1,200	1,718
Factor A =			
$\frac{Cl_B}{Cl_{Ur}} \times \frac{Urea_{Ur}}{Urea_B} \times \frac{\text{Largest fraction (c c)}}{\text{Night volume (c c)}}$	30	25	24

\*Average values obtained by Cutler Power and Wilder in twenty four untreated patients with Addison's disease

## DISCUSSION

The explanation of the abnormal changes in the test in these patients is not clear. There are several possibilities which will be considered. (1) *Abnormal gastrointestinal motility*. In tabes dorsalis there may be episodes of pylorospasm, as well as increased or decreased gastrointestinal activity. Pyloro spasm or decreased gastrointestinal motility would result in gastric retention of the water ingested for the performance of the test. Consequently, intestinal absorption would be decreased and water diuresis delayed, resulting in a positive volumetric part of the test. (2) *Disturbed innervation (decreased tone) of the renal efferent arterioles* may reduce the rate of glomerular filtration and thus produce a delayed diuresis. (3) *Abnormal innervation of the adrenals or actual syphilitic involvement of these glands* is unlikely since normal chloride ion values were obtained in six out of the nine cases. (4) *Impaired intestinal absorption of water* seems unlikely in the absence of diarrhea or dehydration. Nevertheless the rates of absorption may have been decreased. (5) *An abnormal distribution of water in the body* as a cause for delayed diuresis is unlikely in view of the preponderance of normal plasma chloride ion values. It is known that in Addison's disease a positive water test is usually associated with a low plasma concentration of chloride. (6) *Overactivity of the pituitary gland* with excessive production of antidiuretic hormone has been suggested in Addison's disease by Gaunt<sup>4</sup>. It has not been determined, however, that this suggestion holds for Addison's disease or tabes dorsalis. (7) *Morphologic renal changes* with resultant delay in diuresis seem unlikely in the presence of a normal urine analysis in all nine patients. Of course, abnormal variations in the hemodynamics of the kidney may play a role.

Whatever the correct explanation may be for the present findings, it is advisable in performing a Kepler water test, to evaluate the individual values

necessary to compute the factor A from the formula devised by Kepler, Power, and Levy,<sup>2</sup>

$$A = \frac{\text{Blood chloride ion (mg \%)} \times \text{Urine urea nitrogen (mg \%)}}{\text{Urine chloride ion (mg \%)} \times \text{Blood urea nitrogen (mg \%)}} \times \frac{\text{Largest functional specimen (cc)}}{\text{Night volume (cc)}}$$

before accepting the results of the test as an indication of Addison's disease (or adrenal hypofunction)

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of these injections were similar to those of the crude preparations (Table I). The hematocrit rose to approximately the same level but returned to normal levels in a shorter period of time.

TABLE I. A COMPARISON OF THE EFFECTS OF CRUDE HYALURONIDASE (3,000 UNITS PER MG N<sub>2</sub>) AND PURIFIED HYALURONIDASE (30,000 UNITS PER MG N<sub>2</sub>) ON THE HEMATOCRIT OF THE ALBINO RAT.

TIME AFTER INJECTION	7,500 UNITS HYAL (3,000 U/MG N)		7,500 UNITS HYAL (30,000 U/MG N)	
	NUMBER OF RATS	MEAN HEMATOCRIT	NUMBER OF RATS	MEAN HEMATOCRIT*
5 min	5	53.2 ± 4.9	5	45.8 ± 1.7
15 min	5	63.2 ± 2.2	5	65.0 ± 2.7
30 min	5	61.2 ± 4.7	5	60.0 ± 6.2
60 min	5	56.4 ± 3.4	5	55.0 ± 7.0
2 hr	5	51.4 ± 3.6	5	46.1 ± 5.3
4 hr	5	45.0 ± 2.9	5	42.6 ± 2.4
Total number of rat	30		30	

Control mean hematocrit\* (eighty-five rats) 43.7 ± 4.9  
\*Includes standard deviation

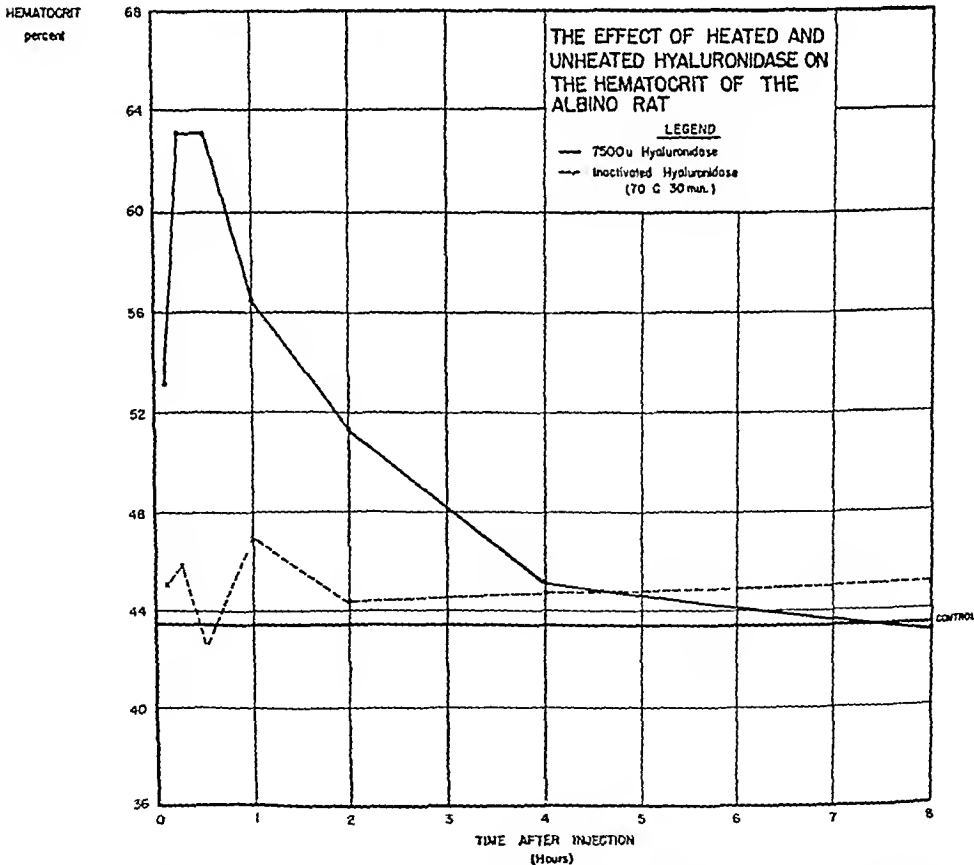


Fig. 2.—The effect of heated and unheated hyaluronidase on the hematocrit of the albino rat.



Suitable control experiments were performed. The testicular extract was heated at 70° C for thirty minutes which completely inactivated the hyaluronidase. A volume of this material equivalent to 7,500 units of hyaluronidase, was administered to thirty five rats. No significant elevation of hematocrit occurred as compared with the eighty five control animals. Striking differences between the effects of heated and unheated testicular extract were evident (Fig 2).

Several other animals were given bovine plasma albumin or histamine intravenously. No equivalent change of the hematocrit was demonstrated.

*Plasma Proteins*—The mean plasma protein level of fifty three control animals was 5.64 Gm per cent. A significant reduction in the plasma proteins occurred in thirty rats receiving 7,500 turbidimetric reducing units of hyaluronidase (Table II). The maximum decrease was noted in fifteen to thirty minutes and thereafter the level of proteins slowly returned to normal. Thirty three animals, to whom were administered heat inactivated hyaluronidase, failed to reveal a similar reduction in the level of the plasma proteins.

Fractionation of the proteins was performed on several samples, but the results failed to demonstrate any significant alteration of the albumin globulin ratio.

TABLE II THE EFFECT OF HEATED AND UNHEATED TESTICULAR EXTRACT ON THE PLASMA PROTEINS OF THE ALBINO RAT

TIME AFTER INJECTION	INACTIVATED HYALURONIDASE*			7 500 UNITS HYALURONIDASE		
	NUMBER OF RATS	MEAN PLASMA PROTEINS (%)†	DEVIATION FROM CONTROL MEAN	NUMBER OF RATS	MEAN PLASMA PROTEINS (%)†	DEVIATION FROM CONTROL MEAN
5 min	5	5.93 ± 0.17	+0.29	5	5.56 ± 0.47	-0.08
15 min	5	6.19 ± 0.33	+0.54	2	4.40	-1.24
30 min	4	5.88 ± 0.22	+0.24	3	4.40	-1.24
60 min	5	5.47 ± 0.25	-0.17	5	4.48 ± 0.27	-1.16
2 hr	4	5.9 ± 0.32	-0.05	5	4.76 ± 0.36	-0.88
4 hr	5	5.56 ± 0.28	-0.08	5	4.65 ± 0.19	-1.01
8 hr	5	5.64 ± 0.26	0	5	5.25 ± 0.55	-0.39
Total number of rats	33			30		

Control mean plasma proteins† (fifty three rats) 5.64 ± 0.43

\* Testicular extract heated at 70° C for thirty minutes. An amount equivalent to 7 500 units of hyaluronidase administered.  
† includes standard deviation

## DISCUSSION

Following the intravenous administration of adequate amounts of testicular extract a prompt direct rise occurred in the hematocrit of the albino rat and the level of plasma proteins was significantly reduced. These changes paralleled the findings of edema, contraction of blood vessels and diffusion of dye described previously,<sup>1</sup> and were further evidence of an increase in capillary permeability.

The disparity between these results and those reported by Chambers<sup>2</sup> as to the effect of hyaluronidase on capillary permeability may possibly be resolved if the amounts of enzyme injected are compared. In order to alter the

TABLE I NUTRITIONAL CONTENT OF NORMAL AND EXPERIMENTAL DIETS

NUTRIENTS	NORMAL DIET	EXPERIMENTAL DIET
Calories	3,170	3,250
Protein (Gm)	70	40*
Carbohydrate (Gm)	330	380
Fat (Gm)	174	175
Calcium (Gm)	0.86	20
Phosphorus (Gm)	1.26	58
Iron (mg)	15.5	12.0
Thiamine (mg)	1.44	54
Riboflavin (mg)	1.84	34
Niacin (mg)	15.6	5.8
Biotin ( $\mu$ g)	44	21.4
L casein factor ( $\mu$ g)	64	22.0
Pantothenic acid (mg)	4.7	1.15
Pyridoxine (mg)	1.76	1.07
Ascorbic acid (mg)	105	90
Vitamin A (I U)	7,400	22,000†

\* Approximately 94 per cent nonanimal

† Mostly  $\beta$ -carotene

Tryptophane was determined microbiologically by the method of Wooley and Sebrell.<sup>1</sup> The term "tryptophane" includes the microbiologically available compounds, such as peptides, closely related to tryptophane that may stimulate the growth of the organisms used. Urine was diluted with water and assayed without further treatment. Samples extracted with ethyl ether to remove indole gave essentially the same values as untreated samples. Storage of samples for one week has no effect on assay values. Food samples were digested enzymatically by pepsin, trypsin, and erepsin. Ba(OH)<sub>2</sub> hydrolysis gave satisfactory checks. Fecal tryptophane determinations were made for a few weeks but showed no consistent results. Nitrogen data were based on the Kjeldahl method.

TABLE II DIET SUPPLEMENTATION SCHEDULE

Period 1	Experimental restricted diet started
Full supplement	B complex supplementation, biotin, 60 $\mu$ g, nicotinamide, 12 mg, pteroylglutamic acid, 90 $\mu$ g, pantothenic acid, 6 mg, riboflavin, 15 mg, paminobenzoic acid, 300 $\mu$ g, pyridoxine, 3 mg, choline 0.5 Gm added
Supplement 3	Thiamine 1.2 mg added
Supplement 4	Protein 45 Gm calcium caseinate added
Supplement 5	Nicotinamide 12 mg, riboflavin 15 mg added
Supplement 6	1 egg for breakfast, 100 Gm (raw weight) ground beef for lunch, $\frac{1}{2}$ pint whole milk for dinner, in order to keep the caloric and protein content similar to that of the other subjects, the calcium caseinate, sugar, and oleo margarine were approximately reduced

## RESULTS

In Fig. 1 are given values for the daily urinary excretion and daily dietary intake of tryptophane. It is apparent that in the case of the five restricted subjects there was no decrease in the amounts of free tryptophane excreted during the periods of restricted dietary intake of proteins and B complex vitamins. This result is in contrast to the excretion of B vitamins which decreased sharply when the dietary intake was reduced.<sup>2</sup> Furthermore no decrease in tryptophane excretion occurred during the period of restricted protein intake despite the intake of large amounts of corn which is deficient in tryptophane.

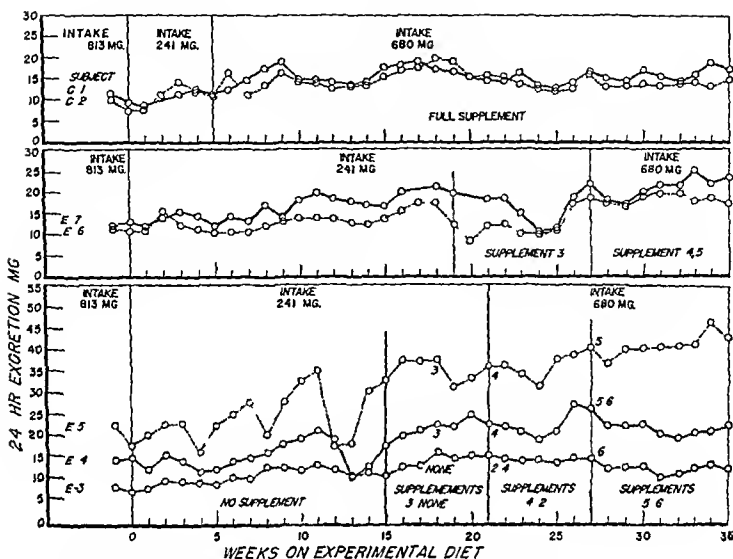


Fig 1—Dietary intake and primary excretion of tryptophane

Ordinates Excretion of tryptophane milligrams per twenty four hours

Abscissas Time on experimental diet weeks. Body weights of subjects pounds C 1 169 C 2 144 E 157 E 6 137 E 150 E 3 183 E 5 146 Dietary intakes as shown Diets and supplements described in text, Table II

During the period of the control diet, the urinary excretion of tryptophane varied from 1 to 3 per cent of the ingested dietary tryptophane. On the restricted dietary intake of tryptophane, the urinary excretion was approximately 3 to 15 per cent of the ingested tryptophane. Actually there was a slight increase in the excretion levels of free tryptophane. The two control subjects showed approximately 5 milligrams increase in daily excretion during the period of supplemented dietary intake as compared with their control period on a normal diet. Perhaps the excretion of metabolic derivatives of tryptophane not micro biologically available was altered by the conditions of the diet and physical activity of the subjects, but these were not studied.

No important differences existed between control and restricted subjects despite the wide difference in protein intake. Variation between individuals is wide as evidenced by the differences between subjects E-3 and E 5. There was no significant loss of body weight during the experimental period nor was any correlation demonstrated between body weight and tryptophane excretion (Fig 1). Nitrogen balance was maintained by all subjects during the period studied (Fig 2). When protein was added to the diet at various intervals each of the subjects receiving this supplement showed an immediate increase in nitrogen excretion over the preceding period of restricted intake.

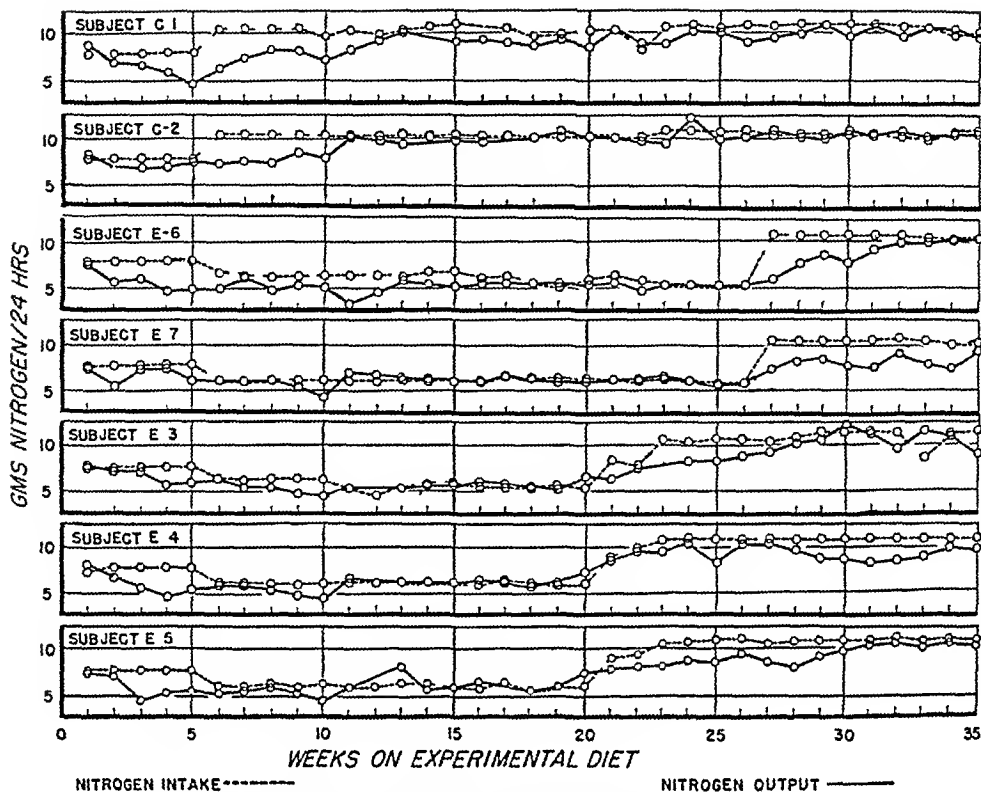


Fig 2—Nitrogen balance of subjects on control and experimental diets From top to bottom two control subjects and five experimental subjects

Ordinates Nitrogen intake grams per day, dotted lines nitrogen excretion grams per day solid lines

Abscissas Time on experimental diet weeks

#### SUMMARY

Seven healthy young men excreted daily in the urine from 8 to 22 mg of free *L*-tryptophane, approximately 1 to 3 per cent of the ingested tryptophane, while on a normal dietary intake of 70 Gm of protein containing 813 mg of free *L*-tryptophane. During twenty-one and twenty-seven week periods on an experimental diet restricted in B complex vitamins and containing 40 Gm of protein (94 per cent nonanimal) and 241 mg of free *L*-tryptophane, five of these same subjects excreted slightly greater amounts of tryptophane than in the control period amounting to a ratio approximately 3 to 15 per cent of the ingested tryptophane. Still greater urinary excretion occurred on a diet supplemented liberally with B complex vitamins and animal protein. Control subjects did not excrete more tryptophane than the subjects on the restricted diet. Nitrogen balance was maintained on levels as low as 0.24 Gm of free tryptophane per day, indicating that the minimum tryptophane requirement of normal adults can be met by a dietary intake of 0.24 Gm daily. No correlation was found between body weight, minimum tryptophane requirement, and excretion of free *L*-tryptophane.

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# ALCOHOL AND PANCREATITIS    SERUM AMYLASE DETERMINATIONS IN NORMAL INDIVIDUALS FOLLOWING INGESTION OF ALCOHOL

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ALCOHOLISM has often been indicted as a possible etiological agent or predisposing factor in pancreatitis, but its actual importance in the production of the disease never has been determined definitely. There is considerable clinical and pathological indirect evidence,<sup>1</sup> however, of such a relationship.

Siegel and Krautman<sup>2</sup> and Gray and co-workers<sup>3</sup> reported lowered serum amylase in patients with acute alcoholism. Domzalski and Wedge<sup>1</sup> in 1948 found that 48 per cent of fifty chronic alcoholic patients admitted to a neuro-psychiatric hospital within forty-eight hours after their last drink had an elevated serum amylase. They postulated a subclinical pancreatitis that might easily be considered clinically to be alcoholic gastritis.

## EXPERIMENT

In an attempt to evaluate further the role of alcohol in relation to pancreatitis or at least as an agent which may affect the values of the serum amylase,<sup>4</sup> an experiment was designed utilizing as subjects fourteen normal, healthy males ranging in age from 27 to 40 years. These men drank habitually only in moderation. After a control serum amylase was determined, from 2 to 12 ounces of a good grade of 86 proof whiskey were ingested within a period of one and one-half hours before a substantial meal. In certain instances of higher alcoholic intake, a serum amylase was determined at this interval. Following the meal, quantities of whiskey varying from 0 to 12 ounces were ingested over a period of four hours. Total amounts of whiskey consumed varied from  $7\frac{1}{2}$  to 24 ounces. Determinations of serum amylase values were made following the period (approximately six hours after the beginning of the experiment) and again on the following day (approximately sixteen hours after the beginning of the experiment). The results are shown in Table I. Subject 4 lost some alcohol through emesis approximately seven hours after starting the experiment. In no instance was there a significant change in the value of the serum amylase.

## SUMMARY AND CONCLUSIONS

Some investigators have noted an increase and others have noted a decrease in the values of the serum amylase in patients suffering from alcoholism. A controlled experiment is described utilizing as subjects fourteen healthy men

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TABLE I CORRELATION OF ALCOHOL INGESTION AND SERUM AMYLASE VALUES

SUBJECT	WHISKEY INTAKE (OZ)			SERUM AMYLASE VALUES			
	FIRST 15 HR	15 HR TO 6 HR	TOTAL INTAKE	CONTROL	15 HR	6 HR	16 HR
1	9	0	9	106		106	88
2	9	9	18	84		<80	
3	9	10.5	19.5	100		106	133
4	12	12	24	<80	<80	89	88
5	7.5	7.5	15	80		100	88
6	6	6	12	80		115	94
7	10.5	9	19.5	<80		<80	<80
8	9	7.5	16.5	84	115	106	100
9	9	4.5	13.5	94		<80	<80
10	7.5	9	16.5	80		84	100
11	9	4.5	13.5	100		133	106
12	7.5	0	7.5	80	94	<80	<80
13	10.5	6	16.5	<80		80	84
14	12	4.5	16.5	<80	90	94	

subsisting on well balanced diets. No significant alterations of the values of the serum amylase were noted during or after the ingestion of alcohol. It would appear that alcohol per se at least in the quantities consumed in the present study, does not affect the level of the serum amylase in the normal individual. It may be that alcohol ingested in the amounts of this controlled experiment are not sufficiently great to affect the serum amylase values or that some other complicating factor present in chronic alcoholic subjects studied by other workers accounted for abnormal values in those instances.

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# STUDIES ON THE DEPRESSION OF BRAIN OXIDATIONS

## I BIOPSY TECHNIQUE AND ANALYSIS OF VARIANCE IN THE SELECTION OF A PENTOBARBITAL CONCENTRATION<sup>1</sup>

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WE ARE pursuing a study of sodium pentobarbital depression of oxygen consumption at various levels in the dog brain. Recently reported spectrophotometric determinations of barbiturates in blood and tissues<sup>2, 4</sup> have not established concentrations of such drugs suitable for *in vivo* experiments. The present investigation was designed to find a concentration of sodium pentobarbital which would significantly, but not completely, depress the oxygen uptake of dog cortex. The experimental design comprises a technique for dog brain biopsy and the application of analysis of variance to standard manometric procedures in a manner which may prove useful in additional experiments with barbiturates and other depressant drugs.

### TECHNIQUE

A cortical biopsy was taken from each of five dogs by a method devised for this experiment. During Nembutal nitrous oxide oxygen anesthesia the temporal muscles were partially excised and the calvarium was removed. The dura was excised and replaced without suturing, which procedure was found necessary to permit access to the cortex at subsequent biopsy without evoking pain and struggling. Number 32 stainless steel wire was employed to close the scalp. Approximately seven days later the incision was reopened during 1 per cent procaine local anesthesia injected into the scalp. The dural flaps and granulations at the periphery were removed. The cortex appeared normal to gross and microscopic examination. A block of cortex approximately 5 by 15 by 20 mm was excised from each hemisphere and placed in a refrigerator.

Slices were prepared for use according to standard manometric practices. The elapsed time from biopsy to oxygenation and equilibration was thirty five minutes. The flasks contained Krebs Ringer phosphate buffer in which the calcium chloride content had been reduced to prevent the precipitation of calcium pentobarbiturate. The treatments comprised duplicate flasks of (1) 0.00, (2) 0.04, (3) 0.08, and (4) 0.12 per cent sodium pentobarbital, as pentobarbital, added to the solution before the flasks were filled. The pH was determined by a Beckman pH meter and was adjusted to 7.4 with 1 per cent HCl. Glucose was added from the side arm at the end of ninety minutes to produce a final concentration of 0.025 per cent. Readings were taken for eight thirty minute periods.

### RESULTS

A, B, and C, Fig 1, are photomicrographs of a section from the biopsy of Dog 2. The section is a representative one and is interpreted as showing no evidence of inflammation, unusual cellular degeneration, or other abnormalities.<sup>6</sup>

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Fig 2 is a graphic representation of the results on Dog 5. Each bar on the graph represents cubic millimeters of oxygen consumed per milligram of tissue dry weight during the preceding thirty minute period. Attention is called to the fact that this is the only expression of the results in terms of dry weight. Fig 2 is derived from Table I by the application of the wet to dry weight ratio which was found to be 5.62. The control for Dog 5 consumed 1.846 cmm of oxygen per milligram of tissue (mean of duplicate samples), wet weight, during the first ninety minutes. Quastel and Whentley<sup>7</sup> reported 0.780 cmm in comparable terms for dog cortex.

Table I presents the data from duplicate samples for four treatments observed through eight thirty minute periods on five dogs. Tables II to VIII are the statistical analysis which is presented in toto to clarify the method. Subsequent statistical analyses from this laboratory will contain a table comparable to Table VII only.

#### DISCUSSION

The dog brain was used as its own control for the *in vitro* addition of the desired drug. This method also allows the dog brain to serve as its own control for *in vivo* administration of a compound. For example it has proved successful at this laboratory to remove tissue from one side controlling the hemorrhage with Gelfoam after which the drug is introduced by the intravenous route before taking a second biopsy from the other hemisphere. Moreover if a small amount of cortex is removed the animal will remain healthy for another procedure upon a different brain level at a later date.

In a departure from custom an analysis of variance<sup>8\*</sup> a statistical method appropriate for small samples has been applied to the data from this experiment. Inasmuch as it is felt that this method of statistical treatment long used in the field of agronomy could find expedient application in many medical researches it will be discussed in detail.

Table II is a two way table for the purpose of calculating the dogs times periods ( $D \times P$ ) mean square in Table VII. It is made up from Table I, for example the figure 1.104 is the total oxygen consumption for all treatments of Dog 1 during Period 1. Likewise Table III for the  $D \times T$  mean square of Table VII contains the figure 3.050 the total oxygen consumption of Dog 1 Treatment 1 for all periods. Table IV referring to  $T \times P$  in Table VII is constructed in similar fashion.

Table VIII traces the calculations. Numbers from the preceding tables have been substituted for algebraic terms so that they may be followed more easily.

The summary of the analysis of variance Table VII contains the deductions which may be drawn from the experiment. The main sources of variation are dogs, periods and treatments. The mean square for each is a representation of the variance in oxygen consumption attributable to its respective source. The first order interaction,  $T \times P$  found to be highly significant, is the appropriate error term for estimating the significance of either treatments or periods.  $D \times T$

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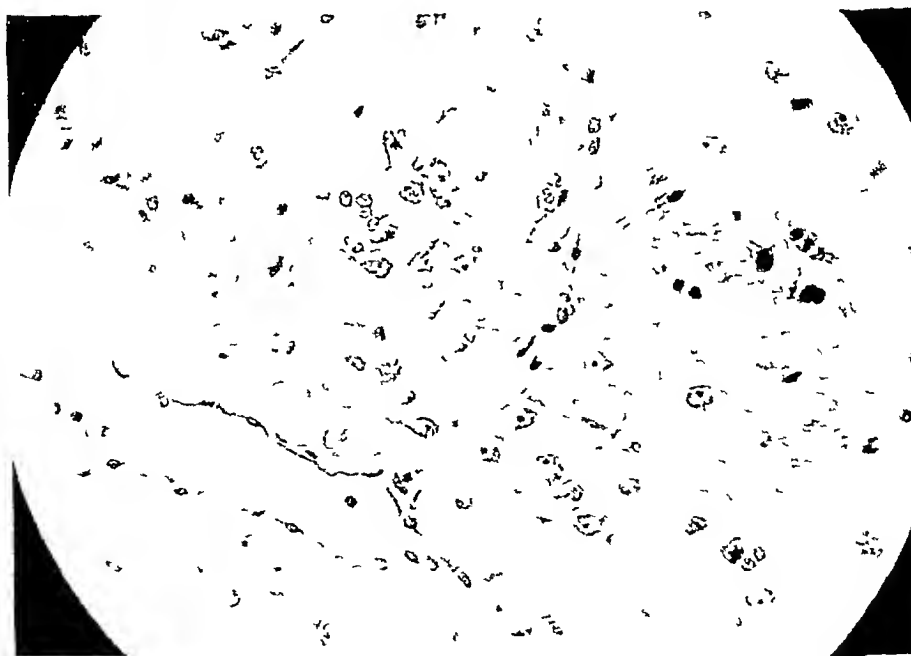
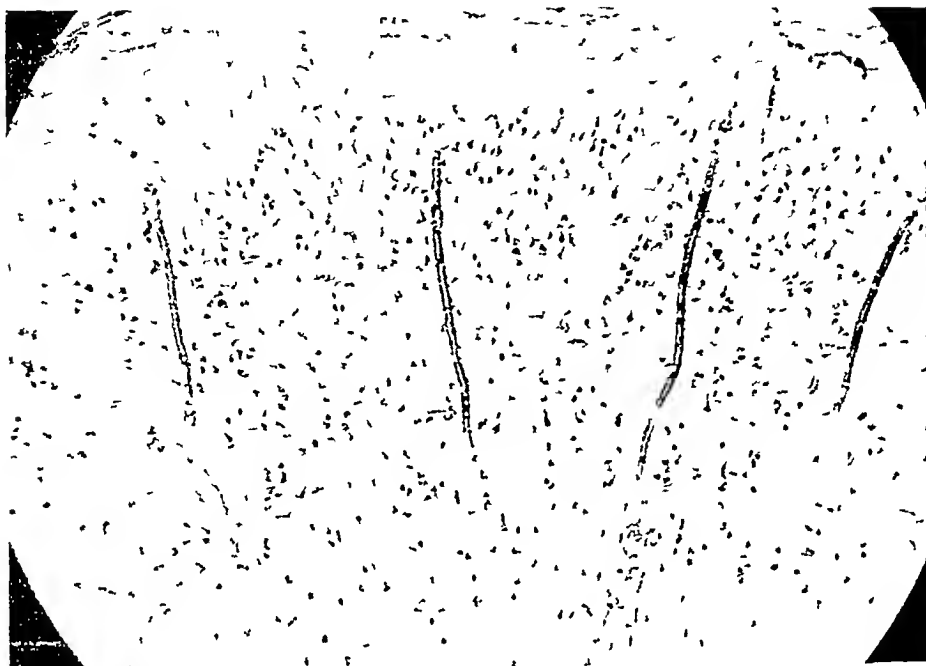
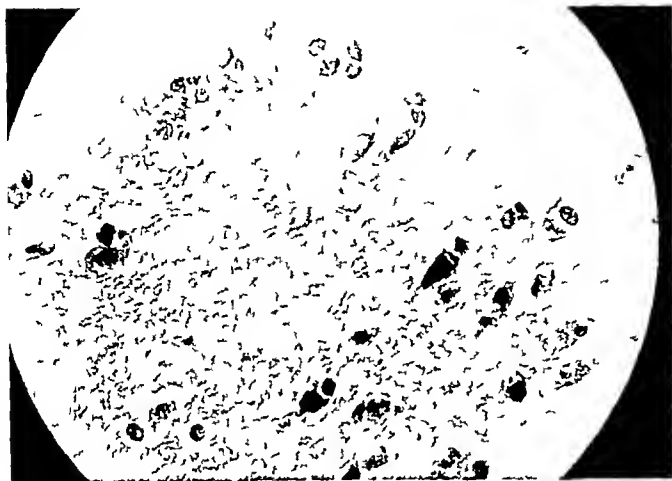
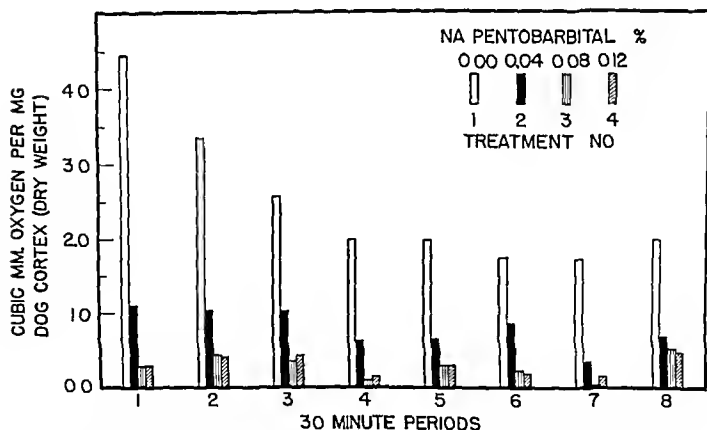
*B*

Fig 1, *A*, *B*, and *C*—Photomicrographs of a section of the cortical biopsy secured from Dog 2 (see text). Magnification of *A*,  $\times 75$ ; *B*,  $\times 250$ ; *C*,  $\times 400$ .



C

Fig 1 (Cont d) —See opposite page for legend

Fig 2 —Graphical representation of oxygen consumption by brain cortex slices from Dog 5  
Glucose in final concentration of 0.05 per cent added at beginning of Period 4

is the error term for dogs. The error terms represent experimental error. Each  $F$  value listed is the ratio of the appropriate mean square to the error term.  $F$  values for significance at the 0.05 and 0.01 levels are included.<sup>8b</sup>

It is of note that the selection of first order interactions ( $T \times P$  and  $D \times T$ ) as measures of experimental error makes the conclusions from this experiment on five dogs applicable to the general dog population, based upon the assumption that the dogs used were a representative random sample of that population. However, use of  $D \times P \times T$  would limit any conclusions to the particular five dogs in the experiment.

It is seen that there is a highly significant difference among the control and other treatments and that the variance among periods is probably significant, as would be expected. The variance among dogs, which approaches significance, is certainly higher than desirable. This fact will be discussed later.

Comparing the control with 0.04 per cent sodium pentobarbital\* (Treatments 1 and 2 in Table VII), one finds the oxygen consumption significantly reduced by that concentration. Furthermore, in comparing 0.04 with 0.08 per cent (Treatments 2 and 3), one may conclude that the inhibition by the former is significantly less than that of the latter. In addition, there is no difference between 0.08 and 0.12 per cent (Treatments 3 and 4). Sodium pentobarbital 0.16 per cent was used on three of the dogs. This oxygen uptake, not shown, was statistically identical with the uptakes of 0.08 and 0.12 per cent. It seems logical to conclude, on the basis of the foregoing facts, that 0.08 and 0.12 per cent sodium pentobarbital cause essentially complete inhibition of dog cortex oxygen consumption.

Quastel's work upon the various substrates inhibited by di-alkyl barbituric acid derivatives was done with a 0.12 per cent concentration of those barbiturates.<sup>9</sup>

Returning to the substantial variance among the five dogs, it is discerned from Table III that the total oxygen consumed by samples from each dog was greater for each succeeding dog used. The linear increase was apparently associated with the improving technique of the experimenters. For example, successively larger sample sizes were used (from 50 to 60 mg. on Dog 1 to 115 to 130 mg. on Dog 5), due to successively larger biopsies. In other words, variation for a form of experimental error has been included with the mean square for a main source of variation, dogs. Deviation from linearity is an appropriate measure of the "actual" variance among the dogs. The mean square (Dogs, Deviation), 0.001117, for this so-called actual variance, that is, the variance inherent in the dogs and uninfluenced by technique, is calculated in Tables V, VI, and VIII<sup>10, 11</sup>. This mean square was found not to be significant when compared with its error term ( $D \times T$ , Deviation).

One more conclusion may be drawn from Table VII. Duplicate samples were sufficient, as indicated by the fact that the mean square for sampling error is of the same magnitude as that for  $D \times T \times P$ .

\*In each instance per cent sodium pentobarbital indicates the concentration present as pentobarbital.

†It will be seen from Table I that a particular pair of flasks was used for each given treatment throughout the experiments. Any differences among flask pairs are thus confounded with treatment effects. It is technically inconceivable that flask pair differences could have been important relative to the large treatment differences so that conclusions concerning treatments are considered valid. In subsequent experiments the samples will be randomized as to placement in flask pairs.

It is perhaps pertinent to point out those things which have been accomplished through analysis of variance and which would have been less clear or less easily attained by other statistical methods. Had Fisher's "T" or the difference of means, both of which are commonly used in medical literature, been employed, the comparison of the full lengths of the curves would have been laborious. Therefore those portions of the curves following the addition of glucose would probably have been totaled, the totals being compared by the alternate methods. Thus the value existing in the varying shapes of the curves would have been lost.

The statistical conclusion regarding the sufficiency of duplicate samples would have been replaced by a merely arbitrary limit of error, such as 10 per cent. Such arbitrary treatment might well have required the use of triplicate samples, which would have unnecessarily reduced the scope of the experiment as delimited by the size of the biopsies.

Moreover, by the other methods mentioned the main sources of variation could not have been simultaneously ascribed their proper proportions in respect to the experiment as a whole. In other words a figure representing variation among dogs could have been computed (for example, a mean and standard deviation of the dog totals). Another figure for the variation among treatments could have been computed and still a third for periods. However one would be left with no clear evaluation of what each might have been while uninfluenced by the others. That evaluation has been accomplished through use of the analysis of variance.

We feel that an even more valid concentration of the drug for *in vitro* use would be that found to occur in the dog brain during hypnosis by the compound. However, sodium pentobarbital in a concentration of the magnitude of 0.04 per cent should prove satisfactory for testing depression of dog brain oxygen consumption at various levels.

#### SUMMARY

A method of dog brain biopsy and an analysis of variance, a statistical method appropriate for small samples, were combined with standard manometric technique in selecting a sodium pentobarbital concentration satisfactory for testing oxygen consumption at various brain levels. The biopsy procedure allows the dog brain to serve as its own control for either *in vitro* or *in vivo* administration of drugs and seems well adapted to this type of experimentation.

An analysis of variance, which permitted the drawing of more conclusions than would otherwise have been available from the data presented, disclosed the following facts:

- 1 Sodium pentobarbital 0.04 per cent, as pentobarbital, significantly depresses the oxygen consumption of dog cortex without completely inhibiting such consumption.
- 2 Sodium pentobarbital 0.08 and 0.12 per cent are quantitatively identical in their almost total abolition of dog cortex oxidations.
- 3 The variation among randomly selected healthy dogs is shown to be statistically nonsignificant for experiments of this type.

4 Duplicate samples were sufficient in the hands of these investigators

Conclusions 3 and 4 would not have been readily deduced without this method of statistical analysis. Conclusions 1 and 2 are much more accessible by this technique than by those more frequently used in the medical literature.

The experiment does not show which substrates were depressed.

TABLE I. CUBIC MILLIMETERS OF OXYGEN CONSUMED PER MILLIGRAM OF DOG CORTEX (WET WEIGHT) DURING EIGHT THIRTY-MINUTE PERIODS FOR EACH OF FOUR TREATMENTS ON FIVE DOGS

TREAT MENT	FLASK	PERIOD								SUM
		1	2	3	4	5	6	7	8	
Dog 1										
1	1	0 298	0 229	0 183	0 275	0 138	0 206	0 046	0 138	3 050
	16	0 406	0 145	0 174	0 174	0 029	0 261	0 174	0 174	
	Sum	0 704	0 374	0 357	0 449	0 167	0 467	0 220	0 312	
2	3	0 109	0 137	0 109	0 082	0 082	0 109	0 027	0 000	1 527
	12	0 245	0 109	0 082	0 109	0 027	0 136	0 082	0 082	
	Sum	0 354	0 246	0 191	0 191	0 109	0 245	0 109	0 082	
3	4	-0 127	-0 021	0 127	0 000	0 021	0 042	0 000	-0 042	0 253
	13	0 092	-0 046	0 023	0 092	-0 046	0 069	0 046	0 023	
	Sum	-0 035	-0 067	0 150	0 092	-0 025	0 111	0 046	-0 019	
4	5	-0 051	0 083	0 034	0 017	0 000	0 051	0 000	-0 017	0 331
	14	0 132	-0 106	0 080	0 027	-0 080	0 106	0 053	0 000	
	Sum	0 081	-0 021	0 114	0 044	-0 080	0 157	0 053	-0 017	
Total sum		1 104	0 532	0 912	0 776	0 171	0 980	0 428	0 358	5 161
Dog 2										
1	1	0 473	0 406	0 254	0 304	0 203	0 254	0 270	0 237	4 881
	16	0 453	0 343	0 453	0 156	0 358	0 215	0 265	0 234	
	Sum	0 926	0 749	0 707	0 460	0 561	0 472	0 535	0 471	
2	3	0 118	0 157	0 103	0 103	0 079	0 079	0 092	0 026	1 541
	12	0 047	0 172	0 109	0 094	0 125	0 062	0 109	0 062	
	Sum	0 165	0 329	0 214	0 199	0 204	0 141	0 201	0 088	
3	4	-0 041	0 096	0 027	0 014	0 028	0 014	0 055	0 000	0 391
	13	-0 043	0 083	0 028	0 042	0 057	-0 014	0 057	-0 014	
	Sum	-0 084	0 181	0 055	0 056	0 085	0 000	0 112	-0 014	
4	5	-0 011	0 068	0 023	0 034	0 023	0 023	0 045	0 000	0 348
	14	-0 102	0 122	0 020	0 000	0 082	0 000	0 041	-0 020	
	Sum	-0 113	0 190	0 043	0 034	0 105	0 023	0 086	-0 020	
Total sum		0 894	1 149	1 019	0 749	0 935	0 636	0 934	0 525	7 161
Dog 3										
1	1	0 565	0 330	0 282	0 173	0 330	0 185	0 267	0 298	5 473
	16	0 653	0 449	0 326	0 316	0 347	0 347	0 296	0 306	
	Sum	1 218	0 779	0 608	0 489	0 677	0 535	0 563	0 604	
2	3	0 188	0 054	0 174	0 054	0 174	0 040	0 080	0 107	1 904
	12	0 186	0 116	0 197	0 070	0 151	0 139	0 046	0 128	
	Sum	0 374	0 170	0 371	0 124	0 325	0 179	0 126	0 235	
3	4	0 044	-0 033	0 055	-0 022	0 065	-0 011	0 011	0 044	0 372
	13	0 039	-0 026	0 077	-0 013	0 039	0 052	-0 013	0 064	
	Sum	0 083	-0 059	0 132	-0 035	0 104	0 041	-0 002	0 108	
4	5	0 048	-0 036	0 045	0 000	0 048	0 000	0 000	0 024	0 360
	14	0 040	-0 040	0 067	0 027	0 027	0 027	0 000	0 080	
	Sum	0 088	-0 076	0 115	0 027	0 075	0 027	0 000	0 104	
Total sum		1 763	0 814	1 226	0 605	1 181	0 782	0 687	1 051	8 109

TABLE I—CONT'D

TREAT MENT	FLASK	PERIOD								SUM
		1	2	3	4	5	6	7	8	
Dog 4										
1	1	0.518	0.420	0.450	0.364	0.224	0.322	0.210	0.266	5.883
	10	0.789	0.594	0.389	0.314	0.67	0.378	0.227	0.151	
	Sum	1.307	1.014	0.733	0.678	0.591	0.700	0.437	0.417	
2	3	0.209	0.185	0.162	0.128	0.116	0.128	0.093	0.070	2.302
	12	0.212	0.212	0.175	0.100	0.150	0.137	0.075	0.150	
	Sum	0.421	0.397	0.337	0.228	0.266	0.265	0.168	0.220	
3	4	0.064	0.075	0.043	0.011	0.021	0.064	0.000	0.021	0.721
	13	0.092	0.079	0.003	-0.033	0.049	0.053	-0.026	0.106	
	Sum	0.156	0.154	0.135	-0.042	0.100	0.117	-0.026	0.127	
4	5	0.079	0.066	0.053	-0.013	0.013	0.079	-0.013	0.013	0.690
	14	0.110	0.069	0.055	-0.027	0.096	0.055	-0.055	0.110	
	Sum	0.189	0.135	0.108	-0.040	0.109	0.134	-0.068	0.123	
Total sum		2.073	1.700	1.319	0.824	1.060	1.216	0.511	0.887	0.596
Dog 5										
1	1	0.678	0.507	0.495	0.398	0.363	0.315	0.303	0.375	7.034
	10	0.000	0.630	0.420	0.310	0.340	0.300	0.310	0.330	
	Sum	1.578	1.197	0.915	0.708	0.703	0.615	0.613	0.705	
2	3	0.180	0.212	0.201	0.117	0.106	0.212	0.064	0.117	2.230
	12	0.209	0.148	0.160	0.098	0.123	0.098	0.062	0.123	
	Sum	0.389	0.360	0.361	0.215	0.229	0.310	0.126	0.240	
3	4	0.030	0.101	0.081	0.030	0.051	0.051	0.010	0.071	0.782
	13	0.069	0.055	0.041	0.000	0.055	0.027	0.000	0.110	
	Sum	0.099	0.156	0.122	0.030	0.106	0.078	0.010	0.181	
4	5	0.032	0.086	0.096	0.043	0.043	0.032	0.021	0.064	0.789
	14	0.074	0.002	0.050	-0.025	0.002	0.025	0.025	0.099	
	Sum	0.106	0.148	0.140	0.018	0.105	0.057	0.046	0.163	
Total sum		2.172	1.861	1.544	0.971	1.143	1.060	0.795	1.299	10.835

TABLE II TOTAL CUBIC MILLIMETERS OF OXYGEN CONSUMED PER MILLIGRAM OF DOG CORTICA (WET WEIGHT) BY ALL SAMPLES FROM EACH DOG DURING EACH THIRTY MINUTE PERIOD

PERIODS	DOGS					SUM
	1	2	3	4	5	
1	1.104	0.894	1.765	2.073	2.172	8.006
2	0.532	1.449	0.814	1.700	1.861	6.356
3	0.812	1.019	1.226	1.319	1.544	5.920
4	0.776	0.749	0.605	0.824	0.971	3.925
5	0.171	0.955	1.181	1.066	1.143	4.516
6	0.980	0.636	0.782	1.216	1.060	4.674
7	0.428	0.934	0.687	0.511	0.795	3.355
8	0.358	0.52	1.051	0.887	1.289	4.110
Total sum	5.161	7.161	8.109	9.596	10.835	40.86

TABLE III TOTAL CUBIC MILLIMETERS OF OXYGEN CONSUMED PER MILLIGRAM OF DOG CORTEX (WET WEIGHT) DURING ALL THIRTY MINUTE PERIODS BY EACH DOG FOR EACH TREATMENT

TREAT MENTS	DOGS					SUM
	1	2	3	4	5	
1	3 050	4 581	5 473	5 883	7 031	26 321
2	1 527	1 541	1 901	2 302	2 230	9 504
3	0 253	0 391	0 372	0 721	0 782	2 519
4	0 331	0 348	0 360	0 690	0 789	2 518
Total sum	5 161	7 161	8 109	9 596	10 835	40 862

TABLE IV TOTAL CUBIC MILLIMETERS OF OXYGEN CONSUMED PER MILLIGRAM OF DOG CORTEX (WET WEIGHT) BY ALL DOGS DURING EACH THIRTY MINUTE PERIOD FOR EACH TREATMENT

PERIODS	TREATMENTS				SUM
	1	2	3	4	
1	5 733	1 703	0 219	0 351	8 006
2	4 113	1 502	0 365	0 376	6 356
3	3 326	1 171	0 594	0 526	5 920
4	2 784	0 957	0 101	0 083	3 925
5	2 699	1 133	0 370	0 314	4 516
6	2 789	1 140	0 347	0 398	4 674
7	2 368	0 730	0 140	0 117	3 355
8	2 509	0 865	0 383	0 353	4 110
Total sum	26 321	9 504	2 519	2 518	40 862

TABLE V COMPUTATION OF DEVIATION FROM LINEARITY OF EACH DOG IN ESTIMATING THE TRUE VARIANCE AMONG FIVE DOGS

DOG	TOTAL	EXPONENT	PRODUCT
1	5 161	-2	-10 322
2	7 161	-1	- 7 161
3	8 109	0	0 000
4	9 596	1	9 596
5	10 835	2	21 670
Sum			+13 783

Sum of (exponent)<sup>2</sup> = 10

Number of observations in each dog total = 64

(Completed in Table VIII)



TABLE VI COMPUTATION OF THE DEVIATION FROM LINEARITY OF DOGS  $\times$  TREATMENTS FOR THE ERROR TERM IN ESTIMATING THE TRUE VARIANCE AMONG FIVE DOGS

DOG	FAPO NFNT	TREATMENT 1		TREATMENT 2		TREATMENT 3		TREATMENT 4		TOTAL
		TOTAL	PRODUCT	TOTAL	PRODUCT	TOTAL	PRODUCT	TOTAL	PRODUCT	
1	1	3030	-6100	1697	-3054	0257	-0500	0331	-0867	
2	1	4881	-4881	1541	-1541	0391	-0391	0348	-0348	
3	0	5473	0000	1304	0000	0377	0000	0360	0000	
4	1	5883	5883	2302	2302	0721	0721	0690	0690	
5	2	7034	14068	2230	4460	0782	1564	0789	1578	
Sum		+8970		+2167		+1383		+1258		+13783

Sum of (exponent) = 10

Number of observations in each total = 10

(Completed in Table VIII)

TABLE VII SUMMARY OF ANALYSIS OF VARIANCE FOR TREATMENTS 1 2 3 4 ON DOGS 1 TO 5

SOURCE OF VARIATION	DEGREES OF FREEDOM	NET SUM OF SQUARES	MEAN SQUARE	F	SIGNIFICANT F AT LEVEL OF	
					0.05	0.01
Dogs	4	0.300181	0.075045	3.012	3.26	5.41
Linear component	1	0.296830	0.296830			
Deviation	3	0.003351	0.001117	0.223	4.07	7.50
Periods	7	0.415564	0.059366	2.093	2.40	3.65
Treatments	3	4.729762	1.576587	55.629	3.07	4.87
D $\times$ P	28	0.235185	0.010185			
D $\times$ T	12	0.297016	0.024751			
Linear component	4	0.277332	0.069334			
Deviation	8	0.039684	0.004961			
T $\times$ P	21	0.035157	0.001674			
D $\times$ T $\times$ P	84	0.151547	0.001804			
Total	159	6.77410				
Sampling error	160	0.300492				
Treatments 1 and 2	1			62.368	4.32	8.02
Treatments 2 and 3	1			10.700	4.32	8.02
Treatments 3 and 4	1			0.00000	4.32	8.02

TABLE III TOTAL CUBIC MILLIMETERS OF OXYGEN CONSUMED PER MILLIGRAM OF DOG CORTEX (WET WEIGHT) DURING ALL THIRTY MINUTE PERIODS BY EACH DOG FOR EACH TREATMENT

TREAT MENTS	DOGS					SUM
	1	2	3	4	5	
1	3 050	4 581	5 473	5 883	7 034	26 321
2	1 527	1 541	1 904	2 302	2 230	9 504
3	0 253	0 391	0 372	0 721	0 782	2 519
4	0 131	0 348	0 360	0 690	0 789	2 518
Total sum	5 161	7 161	8 109	9 596	10 835	40 862

TABLE IV TOTAL CUBIC MILLIMETERS OF OXYGEN CONSUMED PER MILLIGRAM OF DOG CORTEX (WET WEIGHT) BY ALL DOGS DURING EACH THIRTY MINUTE PERIOD FOR EACH TREATMENT

PERIODS	TREATMENTS				SUM
	1	2	3	4	
1	5 733	1 703	0 219	0 351	8 006
2	4 113	1 502	0 365	0 376	6 356
3	3 326	1 474	0 594	0 526	5 920
4	2 784	0 957	0 101	0 083	3 925
5	2 699	1 133	0 370	0 314	4 516
6	2 789	1 140	0 347	0 398	4 674
7	2 368	0 730	0 110	0 117	3 355
8	2 509	0 865	0 383	0 553	4 110
Total sum	26 321	9 504	2 519	2 518	40 862

TABLE V COMPUTATION OF DEVIATION FROM LINEARITY OF EACH DOG IN ESTIMATING THE TRUE VARIANCE AMONG FIVE DOGS

DOG	TOTAL	EXPONENT	PRODUCT
1	5 161	-2	-10 322
2	7 161	-1	- 7 161
3	8 109	0	0 000
4	9 596	1	9 596
5	10 835	2	21 670
Sum			+13 783

Sum of (exponent)<sup>2</sup> = 10

Number of observations in each dog total = 64

(Completed in Table VIII)

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## STUDIES ON SERUM ESTERASE

### THE SERUM ESTERASE IN LIVER DISEASE

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GOMORI'S recently described micromethod for the determination of blood esterase<sup>1</sup> gave occasion to examine this enzyme in human sera under various clinical conditions. The principle of this method is that phenyl benzoate is hydrolyzed into phenol and benzoic acid when incubated with an esterase containing material. The phenol is then linked to a chromogen—Red B Salt—which permits quantitative colorimetric estimation. The amount of enzymatic activity of the substrate is expressed in micromoles of phenol per cubic centimeter material. The details of the procedure are described in Gomori's original publication.<sup>1</sup> The method can be carried out easily in a clinical laboratory.

Our series consists of more than 400 estimations performed with sera from 150 unselected patients of this hospital. The blood was withdrawn in the morning before breakfast and was permitted to clot, the serum was separated and used either immediately or after storage in the refrigerator for twenty-four hours. Many of the patients were examined on several occasions.

Fig. 1 shows graphically the values obtained. The patients are enumerated on the abscissa, the esterase values indicated on the ordinate. Each dot represents one estimation. Where more than one estimation was done on the same patient, the individual values are connected by a vertical line. From this graph it is evident that

- 1 Most of the values fall within a range between 12 and 25 micromoles, with an average of 16 micromoles.

- 2 The fluctuations of serial estimations in the same individual are extremely wide in many instances.

- 3 There are a number of estimations, individual and serial, which are far below the average range.

The wide spread of the values of the same individual may suggest that the blood esterase fluctuates rapidly and widely under physiologic conditions and therefore may have little clinical significance. Since this survey was done on hospital patients, the alternative possibility had to be considered that the fluctuation of the values paralleled in some way the change in the patients' illnesses. Thus it became advisable to determine the blood esterase level in

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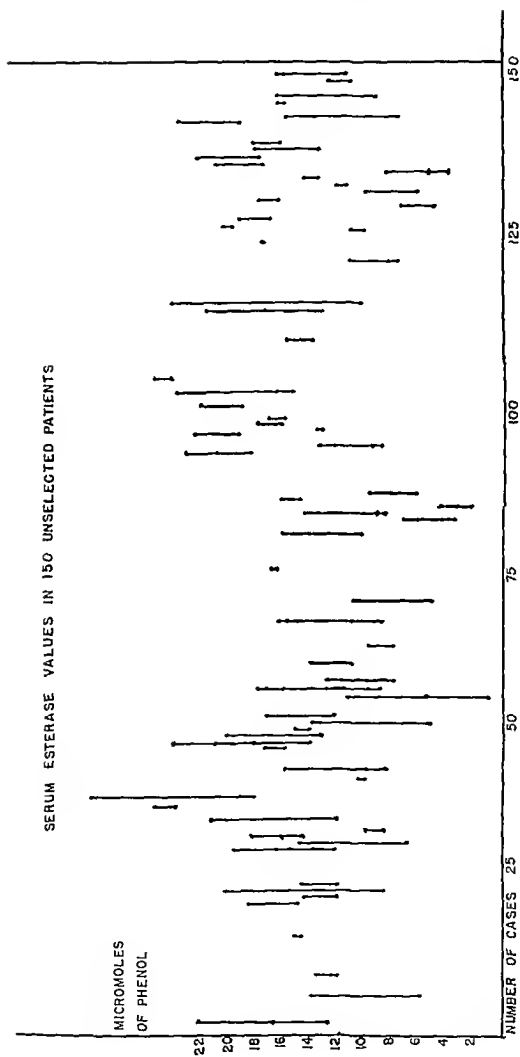


FIG. 1

healthy control persons. This was done in five individuals who were followed with serial estimations over periods up to thirty days. It was found that each test person maintained a rather constant serum esterase level, although the different individuals showed different degrees of enzymatic activity in their serum, all of them were, however, well within the average range. Fig 2 demonstrates the serum esterase levels of three normal individuals during a ten-day period. In another series the effect of absorption

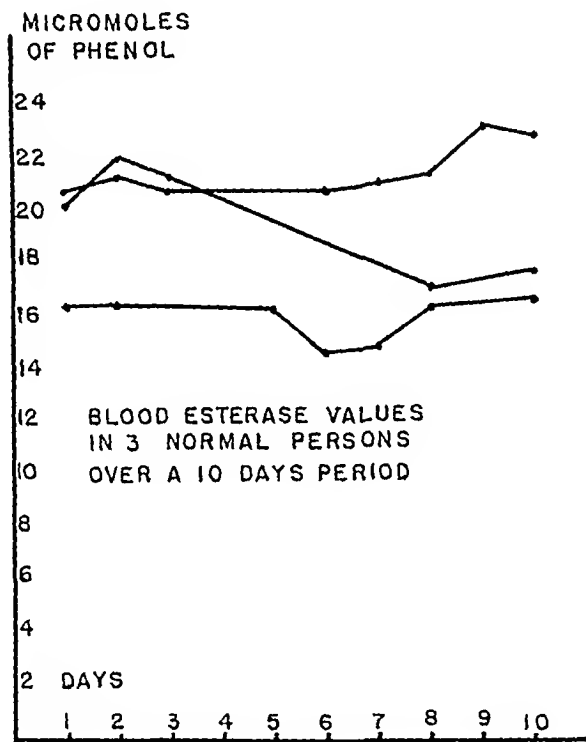


Fig 2

and digestion of a fatty meal upon the normal serum esterase level was determined by hourly estimations over an eight-hour period. No significant fluctuations were found. Thus the stability of the serum esterase in health appeared to be established.

We proceeded to correlate the clinical diagnoses of our patients with their blood esterase values. At the same time all those patients were selected who showed an enzymatic activity of less than 10 micromoles phenol. This level was set arbitrarily in the hope that an analysis of values far below the average might yield positive results and reveal some clinical condition associated with or even responsible for such decrease of enzymatic activity of the serum.

A tabulation of the clinical diagnoses of our 150 patients is given in Table I. The pathologic conditions represented in this series represent cardiovascular disturbances, gastrointestinal diseases, tumors, chronic pulmonary

diseases such as tuberculosis, pulmonary fibrosis, and bronchiectasis, acute inflammatory conditions, liver diseases, chronic pancreatitis, diabetes, thyroid diseases, arthritis, and a few miscellaneous conditions as fractures severe burns, bilateral polycystic kidneys and pyelitis with the differential diagnosis of amyloidosis. Twenty seven of these patients showed a serum esterase value below 10 micromoles at a single determination or several determinations. Fifteen low values were found in the group of liver diseases, that is, more than half of this group was in the low range. Twelve more low values occurred in other pathologic conditions: four in chronic pulmonary diseases, two in gastrointestinal diseases, and one each in the tumor group, in the cardiovascular group, and in the diabetic group. The three remaining low values were found in the patients with severe burns, bilateral polycystic kidneys and chronic pyelitis with possible amyloidosis.

TABLE I. THE DIAGNOSES AND THE DISTRIBUTION OF LOW SERUM ESTERASE VALUES IN 150 UNSELECTED PATIENTS

DIAGNOSIS	NUMBER OF PATIENTS		
	TOTAL	SERUM ESTERASE	
		ABOVE 10 MICROMOLES	BELOW 10 MICROMOLES
Liver disease	28	13	15
Cardiovascular disease	14	13	1
Chronic pulmonary disease	30	26	4
Gastrointestinal disease	18	16	2
Acute inflammatory condition	8	8	0
Malignant tumor	10	9	1
Neuropsychiatric condition	11	11	0
Chronic pancreatitis	5	5	0
Diabetes mellitus	11	10	1
Thyroid disease	4	4	0
Arthritis	5	5	0
Miscellaneous (fractures severe burns, bilateral polycystic kid- neys, amyloidosis?)	6	3	3
	150	123	27

An analysis of the case histories of these thirteen patients with extra-hepatic diseases showed the following: the four pulmonary patients had far advanced bronchiectases (two) or pulmonary tuberculosis, three of them had just undergone chest surgery when the low values were obtained, all four were in poor nutritional state. The two gastrointestinal patients had peptic ulcers with acute complications due to pyloric obstruction in one and perforation with peritonitis in the other. Both patients had serum esterase values above 10 micromoles in the postoperative recovery phase. The cardiovascular and the diabetic patient had enlarged livers but the usual liver function tests had failed to show any abnormality. The patient with tumor was suffering from Hodgkin's disease and was under treatment with nitrogen mustard when his serum esterase was determined. Only one single estimation had been done in this patient. In these patients as well as in the one with extensive burns and the other with the differential diagnosis of amyloidosis, it seems doubtful whether the low serum esterase levels can be ascribed to

their organ disease or are due to some general metabolic involvement. The great majority of patients with cardiovascular diseases, pulmonary conditions, tumors, ulcer disease, and diabetes mellitus had serum esterase levels well above 10 micromoles. It should be noted also that none of the five patients with chronic pancreatitis had shown a low serum esterase activity, one patient in this group had undergone a subtotal pancreatectomy.

Thus, it appeared that only the group with liver diseases was characterized in the majority of cases by a low level of serum esterase. A differential diagnostic breakdown of this group showed sixteen cases of portal cirrhosis, five cases of infectious hepatitis, six cases of cholangitis, cholecystitis, and cholelithiasis, and one case of biliary xanthomatosis (Table II). The low values were confined to cirrhosis of the liver and to the one case of biliary xanthomatosis. In two instances of liver cirrhosis, where the diagnosis had

TABLE II THE DIFFERENTIAL DIAGNOSIS AND DISTRIBUTION OF LOW SERUM ESTERASE VALUES IN 28 PATIENTS WITH LIVER DISEASE

DIAGNOSIS	NUMBER OF PATIENTS		
	TOTAL	SERUM ESTERASE	
		ABOVE 10 MICROMOLES	BELOW 10 MICROMOLES
Portal cirrhosis	16	2	14
Biliary xanthomatosis	1	0	1
Infectious hepatitis	5	5	0
Cholangitis / cholecystitis	6	6	0
	28	13	15

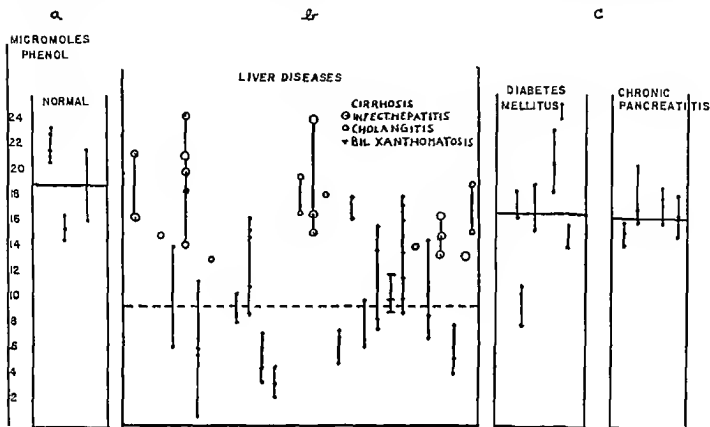
been established by history, clinical findings, and the usual liver function tests, the serum esterase levels were above 10—in one instance 12, in the other 16.2 and 18. Out of the fifteen cases with low levels, the usual liver function tests such as cephalin flocculation, thymol turbidity, total serum proteins, and albumin-globulin ratio had been done and had confirmed the diagnosis in thirteen instances. In one patient the differential diagnosis of infectious hepatitis had been entertained for some time but while the jaundice was clearing he developed ascites, low blood proteins, and a reversal of the albumin-globulin ratio and there could be little doubt that the infectious hepatitis was superimposed upon a cirrhotic process. The serum esterase level had been low since his admission to the hospital. The second patient was in cardiac failure, the liver was large and hard, no liver function tests had been done, the serum esterase level was low and decreased progressively. The day prior to his death we found an activity of only 0.96 micromole phenol in his serum, the lowest of all our values. The autopsy revealed in addition to other findings a far-advanced cirrhosis of the liver. Fig 3, b demonstrates the individual values obtained in all patients with liver disease.

A direct relationship between course of the disease and the serum esterase level was found in several other patients. Two other patients with cirrhosis died during the period of our investigation, both showed progressively decreasing esterase values and again the lowest levels were found shortly prior to death.



On the other hand, in patients who improved under medical management, the serum esterase increased gradually and paralleled the improvement of the usual liver function tests and the general condition of the patient.

The average value for all levels obtained in cirrhosis of the liver is 9.5 micromoles phenol. Such average may be of little importance for differential diagnostic clinical purposes because of the spread of the individual values.



Fig

It demonstrates, however, a significant difference from the average value for healthy persons, diabetic subjects, or patients with chronic pancreatitis in whom it lies between 16 and 19 micromoles.

#### COMMENT

Gomori<sup>1</sup> found the serum esterase activity ranging between 14.5 and 41.2 micromoles per cubic centimeter in his series of fifty-one unselected patients. The majority of our values is well within these limits. Gomori did not give a list of the clinical diagnoses of his patients nor did he indicate the range of fluctuations of enzymatic activity in the serum of the same individual at repeated examinations. While these variations have been extremely wide in many of our patients we have found that in health the serum esterase level tends to be rather constant.

A markedly low esterase activity in the serum of patients with chronic liver disease has been reported by a number of other investigators.<sup>2,3</sup> These studies were done with different methods, the stalagmometric or the acid titration procedure. Further investigations will have to show whether the esterases determined by these procedures are identical. Lagerloef found that the level of the serum esterase is not altered by pancreatectomy and postulated that this enzyme originates in the liver. Our own observations are in

agreement with his findings. While chronic pancreatic disease such as chronic pancreatitis and pancreatic sclerosis with steatorrhea was not associated with low serum esterase values, such decreased enzymatic activity was present in almost all of our patients with chronic and extended parenchymatous liver damage.

Our series is too small to offer the serum esterase estimation as another liver function test or as a diagnostic test for cirrhosis of the liver. Most of our patients had far-advanced disease and we do not know yet whether significant changes in the serum esterase can be detected early enough to be of diagnostic help and to supplement the already large battery of liver function tests.

In view of our observations of low serum esterase levels in a few patients with chronic pulmonary disease, it is of interest that Paraf and associates,<sup>6</sup> working with the stalagmometric method, reported a direct relationship between serum esterase levels and the severity of pulmonary tuberculosis. An analysis of the values of all our pulmonary cases, however, did not reveal such relation. We found low values in only four out of thirty patients, three of them had just undergone severe thoracic surgery. It seems that their general condition, possibly an involvement of liver metabolism, was responsible for the decrease in the serum esterase activity and not the underlying pulmonary disease.

Nothing is known about the physiologic function of the serum esterase determined with the described method. Our observations, however, seem to indicate that this enzyme is present in the blood of healthy individuals at a rather constant level that it is not involved in the process of absorption or digestion of fatty nutrients and that it is not related to the fat splitting enzyme of the pancreas.

#### SUMMARY

1 The Gomori method for estimation of the serum esterase was used for the examination of the serum of 150 unselected patients and a group of normal individuals.

2 Under physiologic conditions this esterase seems to be present in the serum at a rather constant level.

3 Under pathologic conditions, mainly in advanced cirrhosis of the liver, this serum esterase is significantly decreased.

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## LABORATORY METHODS

### THE CONVERSION OF A STANDARD INCUBATOR TO A CARBON DIOXIDE INCUBATOR

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AN ATMOSPHERE of increased CO concentration has been proved many times to be an important factor in stimulating the growth of a number of bacteria. The use of candle jars is one of the simplest means of obtaining additional CO and was the method employed for several years by the Georgia Department of Public Health. This method had many disadvantages which were especially evident in the cultivation of blood specimens for *Brucella* organisms. These specimens were examined weekly and the negative cultures incubated for four weeks. The long incubation period necessitated the use of many jars the handling of which was time consuming and the additional moisture from the candles encouraged fungous contaminants. A concentration of 5 to 10 per cent CO<sub>2</sub> has been shown to produce optimum growth of *Brucella abortus*.<sup>1</sup> Since the CO concentrations in the candle jars were usually less than this level and also quite variable, some device was needed to simplify and improve this method. The device had to be simple to operate, efficient and large enough to accommodate both the *Brucella* cultures and those of other organisms requiring increased CO atmosphere.

Various methods have been used by other laboratories. Large air tight cans or jars have been fitted with special outlets so that the desired percentage of air could be removed and replaced with CO<sub>2</sub>. After adding the CO<sub>2</sub>, these cans or jars were incubated in an ordinary air incubator. Special incubators have been constructed such as the nonventilated "capnic incubator." This incubator is filled by means of a rotameter, with a measured mixture of gases producing the desired atmosphere. Another procedure is the use of a regular air incubator into which a very small amount of CO<sub>2</sub> is allowed to flow constantly. This latter method is very inaccurate as the percentage of CO in the incubator is unknown. Our plan was to pass a measured amount of CO into an ordinary air incubator once a day and then by testing samples of the incubator air at hourly intervals to determine the decrease in CO over a twenty four hour period. By this method we hoped to arrive at the amount of CO which should be added once a day to give an atmosphere of 5 to 10 per cent during most of the following twenty four hours.

From the Georgia Department of Public Health Laboratories.

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## CONSTRUCTION AND OPERATION

An air incubator\* was selected. The two lower ventilation outlets on each side were closed from the inside with cork stoppers and a two-hole rubber stopper was placed in each of the two top outlets. One hole in each of the stoppers was left open for ventilation, and in the other two holes were placed glass tubes for the addition of  $\text{CO}_2$  (Fig 1, *A*) and for sampling the incubator air (Fig 2, *L*).

We use the Liquid Carbonic Corporation's Medical Carbon Dioxide cylinder with a regulator made by the same company, but presumably any type  $\text{CO}_2$  cylinder with a screw valve regulator would be satisfactory. The usual dial gauge found on these regulators is not shown in Fig 1. Such an indicator is unnecessary as it does not register less than 5 pounds pressure. Valve *E* is also unnecessary except in the initial opening and checking of the regulator, in routine use it is kept open at all times.

When  $\text{CO}_2$  is to be added, the cylinder valve (*B*) is opened all the way and the screw valve (*C*) is carefully closed until the desired pressure is obtained. This pressure is measured by mercury in a U tube (*D*), the open side of which is marked in fractions of an inch. This U tube is connected to the regulator by a T tube, the other side of which is connected with the glass tube (*A*) that takes  $\text{CO}_2$  into the incubator. The U tube is supported in a large plugged test tube that is clamped to the regulator. (This clamp is not shown in Fig 1).

The apparatus for measuring the percentage of  $\text{CO}_2$  in the incubator is a homemade modification of an Orsat gas apparatus (Fig 2). Samples of gas are taken through a tube (*L*) at various levels in the incubator, the quantity of the samples being controlled by clamp *II*. With clamp *II* open and clamp *K* closed, the gas is brought into the burette (*Y*). This burette is made from the bulb of a 50 ml pipette attached to a long glass tube. The tube is calibrated in milliliters and the total measurable capacity of the burette is determined (in this apparatus from *X* to *Y* is 58 ml). By means of a leveling bottle (*N*), the burette is filled with distilled water, to which has been added 2 drops of concentrated  $\text{H}_2\text{SO}_4$  and sufficient methyl red to show that the water is acid at all times. The absorption gas pipette (*M*) is made by attaching the bulbs from two 100 ml pipettes and filling the absorption compartment with pieces of glass tubing. To the crooked neck of the expansion compartment is attached a gas expansion bag, in this case made from a small funnel covered with rubber from a rubber glove (*R*). The pipette is filled with approximately 80 ml of 50 per cent KOH.

To make a reading of percentage  $\text{CO}_2$  with this apparatus, the KOH is first brought up to *W* by lowering the leveling bottle below the table top with *II* closed and *K* open. When the KOH is at *W* (a point about midway between the bulb and the rubber tubing) *K* is closed. Then by opening *II*, the burette is filled and emptied twice with gas from the incubator, to remove any gas left from previous tests. About five minutes is allowed after each of these fillings,

\*Theleo Precision No 6 Electric incubator No 1485 large

then the sample (58 ml) of  $m$  is carefully taken by lowering the water from  $X$  to  $Y$ . The tube is allowed to drain for one minute, then the water in the bottle and that in the tube are leveled at  $Y$ , and  $H$  is closed.  $K$  is opened

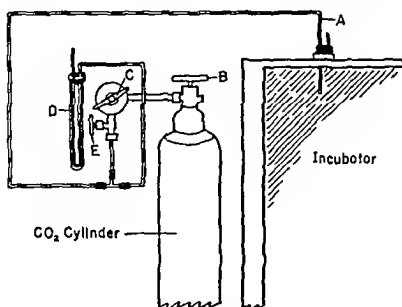


Fig 1 Apparatus for adding carbon dioxide to the incubator  
(As used routinely)

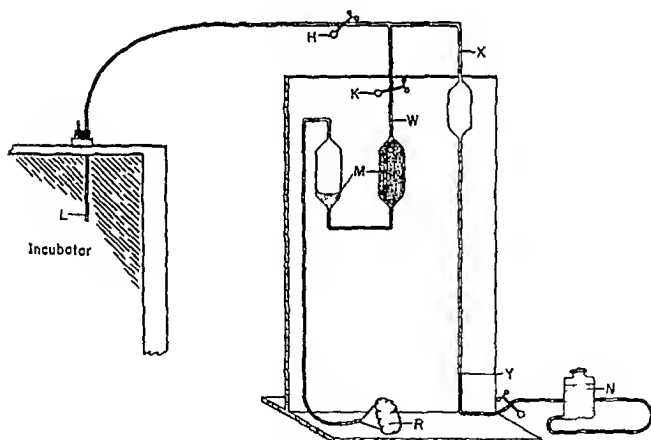


Fig 2 Apparatus for testing carbon dioxide content of the incubator  
(Used only while determining amount of CO<sub>2</sub> required)

and the gas is forced into the gas pipette ( $M$ ) by raising the leveling bottle until water reaches  $X$ . This forcing of the gas into the pipette is repeated six times, with care taken not to force bubbles through the KOH. After the sixth

absorption the KOH is brought again to the point W, and K is closed. The tube is allowed to drain one minute, then the water in the bottle is leveled with that in the tube and a reading is made. The number of cubic centimeters of water above the mark Y represents the quantity of CO<sub>2</sub> absorbed from the sample of gas. This is transcribed from milliliters of CO<sub>2</sub> in 58 ml of gas to percentage of CO<sub>2</sub> in the incubator at the level and time tested.

As in working with any Oisat gas apparatus, several precautions should be observed. When fresh water or fresh KOH is added, air is forced through the apparatus several times so that all dissolvable air will be dissolved by the liquids before any test is made. Periodically the absorption of the gas by KOH is checked by forcing the gas being tested into the pipette seven to eight times and comparing that reading with the reading made after six absorptions. In no instance was this higher than the reading after six absorptions.

#### ADDITION OF CO<sub>2</sub>

The gas-measuring apparatus was used to determine the amount of CO<sub>2</sub> required to give an atmosphere of approximately 5 to 10 per cent in the incubator over the longest practical period. One hundred and thirty-nine readings were made. The first few determined what percentage of CO<sub>2</sub> would be obtained by holding the mercury column at different heights for varying lengths of time. Then several tests were made to determine the approximate drop in CO<sub>2</sub> during the first few hours. These tests showed that if approximately 20 per cent is added there will be a drop to approximately 12 per cent in three hours, and then the decrease will be markedly less and more constant. With this tentative information it was decided to add 1 in mercury of CO<sub>2</sub> for four and a fourth minutes. Several series of hourly readings were made starting fifteen minutes after the addition of CO<sub>2</sub> and extending for twenty-four hours. The average percentages obtained are shown in Fig. 3. In thirteen series the average initial percentage of CO<sub>2</sub> was 19, and hourly readings for the next three hours were 14.6, 13.1, and 11.7 per cent respectively. At this point the rate of decrease level to only 0.35 per cent per hour for the next twenty-one hours tested, leaving 4.4 per cent CO<sub>2</sub> in the incubator at the end of twenty-four hours. Variations from these averages were small. In all the readings for the last twenty-one hours, the highest decrease in CO<sub>2</sub> per hour was 0.5 per cent and the lowest, 0.2 per cent. Although there was considerable fluctuation of the mercury column at various times while adding the CO<sub>2</sub>, in no instance was there a variation of over 1.7 per cent in the incubator fifteen minutes after the addition, and at the end of three hours the variation was never over 0.5 per cent. From these readings it may be concluded that after adding 1 in mercury of CO<sub>2</sub> for four and one-fourth minutes the incubator tested will contain approximately 11.7 to 4.4 per cent CO<sub>2</sub> for twenty-one hours. This concentration range is apparently favorable to the growth of *Brucella abortus* and to most of the other organisms that require increased CO<sub>2</sub> tension.

The readings, as recorded in Fig 3, were of samples of air taken from the middle shelf of the incubator. Two series were observed hourly on samples from the lowest shelf and they showed approximately the same percentage as the middle shelf. Air from the top shelf also was tested in the same manner, the initial readings, fifteen minutes after addition of  $\text{CO}_2$ , were approximately 4 per cent lower than those of the middle and lowest shelves, but after one hour the percentages were the same. Three series also were done on a candle jar, the initial readings, five minutes after the candle stopped burning, were

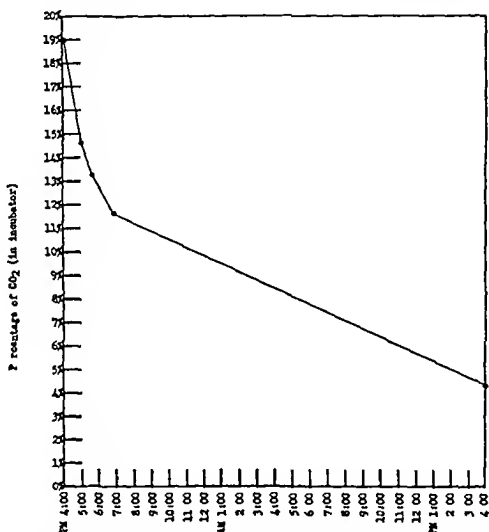


Fig 3 --Rate of  $\text{CO}_2$  decrease in the incubator after adding 1 in mercury of  $\text{CO}_2$  for four and a fourth minutes (incubator kept closed). These are the averages from thirteen series of hourly readings.

3.1, 3.1, and 2.8 per cent. The percentage of  $\text{CO}_2$  in the jar showed a steady decrease to a fraction of 1 per cent at the end of twenty-four hours, but work was not done to show what the decrease in the jar would be in the presence of *Brucella* organisms or contaminants.

Routinely,  $\text{CO}_2$  is added at about 4:00 PM and the incubator doors are kept closed until 2:00 PM the following day, at which time the transplants and examinations are made. If for any reason the doors must be opened in the morning, a small amount of  $\text{CO}_2$  may be added so that the incubator will not be without  $\text{CO}_2$  for the remainder of the day. Before each addition of  $\text{CO}_2$  the incubator doors should be opened to remove any  $\text{CO}_2$  left from the previous addition. Addition of  $\text{CO}_2$  does not alter the incubation temperature.

## CULTURES

Eight strains of *B. abortus* were used and twenty eight comparisons made to show the difference in growth in candle jars and the CO<sub>2</sub> incubator. Some of these strains were recently isolated and others had been on artificial media for several months. Bottles of Tryptose broth were inoculated and incubated for forty-eight to seventy-two hours, then two sets of decimal dilutions were made through twenty tubes on each culture. One set was incubated in the CO<sub>2</sub> incubator and the other in candle jars. Observations for turbidity were recorded at the end of forty-eight hours. After that time, accurate comparisons could not be made because in most instances tubes in the incubator showed heavy growth through the twentieth dilution. In seventeen of the twenty eight comparisons the candle jars were used as in routine Brucella culturing, that is, the candles were lighted when the tubes were first placed in the jars and the jars were not opened until after forty eight hours of incubation (Fig 4). A second group of eleven comparisons was made in which the candles were re-lighted at twenty-four hours (Fig 5).

In both groups the incubator apparently gave better growth than the candle jars, but the differences were much more evident when the candles were not re-lighted. The average difference in growth in the incubator and the jar as used routinely (Fig 4) was approximately nine decimal dilutions more in the incubator, with the greatest difference being nineteen tubes and the least four tubes. When the candles were re-lighted at twenty-four hours, the average difference in eleven comparisons was four decimal dilutions more in the incubator. In four instances the comparative growth did not differ more than one tube, and in two instances the incubator produced growth in ten decimal dilutions higher than the candle jar (Fig 5).

For seventeen months all cultures for Brucella and then transplants have been grown in the CO<sub>2</sub> incubator. During this time Brucella organisms have been isolated from fifty-six specimens. From one hundred and eight blood clots cultured from ninety three patients having positive agglutination reactions with Brucella antigen twenty positive cultures were obtained. Ten of these were *B. abortus*, nine, *B. suis*, and 1, unclassified. From seventy six special bottles containing culture media<sup>3</sup> sent to physicians for whole blood specimens, fifteen *B. abortus* and twenty-one *B. suis* cultures were isolated. During this routine use of the incubator, mold contaminants were observed very rarely. These contaminants had been quite prevalent in cultures kept in candle jars for several weeks.

Before using the CO<sub>2</sub> incubator for routine cultivation of gonococci, several series of duplicate plantings were made from cervical specimens in order to compare growth (number and size of colonies) obtained in the CO<sub>2</sub> incubator with that in candle jars. Growth was approximately the same, and in no instance was a culture positive in the candle jar and negative in the CO<sub>2</sub> incubator. Since these comparisons proved satisfactory the CO<sub>2</sub> incubator was put into routine use for growing all gonococcus cultures and has been used satisfactorily for seven months. Other miscellaneous cultures (streptococci, *Hemophilus m-*



*fluenzae*, and *Streptobacillus moniliformis*) have been grown successfully in the incubator. For routine culture work it has proved to be convenient and inexpensive to operate.

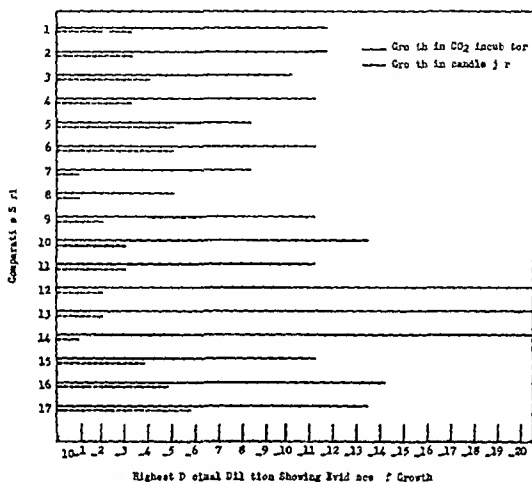


Fig. 4—Comparative growth of *Br. abortus* in broth incubated in the CO<sub>2</sub> incubator and in candle jars (Jars not opened until after forty eight hours). Growth is recorded at forty eight hours.

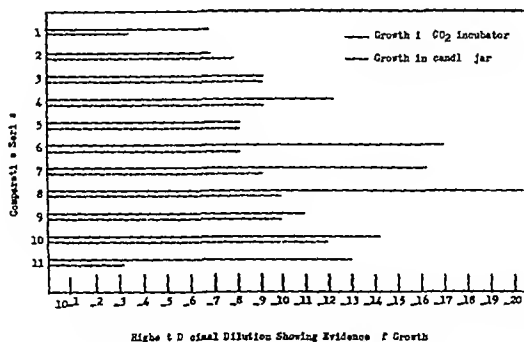


Fig. 5—Comparative growth of *Br. abortus* in broth incubated in the CO<sub>2</sub> incubator and in candle jar (candles relighted after twenty four hours). Growth is recorded at forty eight hours.

## SUMMARY

The conversion of an ordinary air incubator to an incubator with a measured increased CO<sub>2</sub> atmosphere has been described. The conversion and standardization were simple and required only materials found in the usual bacteriologic laboratory. After adding 1 in. mercury of CO<sub>2</sub> for four and a fourth minutes, this incubator maintains an approximate percentage of 11.7 to 4.4 per cent of CO<sub>2</sub> for twenty one hours and has proved to be more efficient than candle jars for growing *Br. abortus* and other organisms requiring an increased CO<sub>2</sub> atmosphere. It is efficient, easy to operate, and practical for daily use in diagnostic laboratories.

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## A PHOTOMETRIC MODIFICATION OF THE HYPOBROMITE METHOD FOR NONPROTEIN NITROGEN

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RAPPAPORT and Eichhorn<sup>1</sup> have recently published a rapid titrimetric micro-method for the determination of nonprotein nitrogen, based upon the hypobromite reaction. They show excellent agreement with the results of micro-Kjeldahl analyses. The application of the hypobromite method to the determination of urea in blood and urine has been discussed by Peters and Van Slyke,<sup>2</sup> who pointed out that urea does not react quantitatively with the reagent, and that ammonia, ureic acid and creatinine do react to some extent. Thus good results in the determination of urea depended upon a balancing of errors.

In a preliminary examination of the Rappaport and Eichhorn method for nonprotein nitrogen in blood filtrates, we found that urea reacted quantitatively, amino acids gave considerably greater than 100 per cent recovery and creatinine, ureic acid or adenine much less than theoretical recovery of total nitrogen. Titration of actual blood filtrates gave values corresponding to 93 to 94 per cent of the total nitrogen, as compared with micro-Kjeldahl analyses.

The present report describes a modified procedure in which the liberated iodine is estimated photometrically and in which reliable and precise results are conveniently obtained.

### METHOD

The reagents were those described by Rappaport and Eichhorn, unless modified as indicated below. All reagent solutions were made up in redistilled water or Vaeoliter water in order to avoid the possibility of turbidity in the photometric analysis. Accurate volumetric flasks and pipettes were used throughout.

#### Reagents—

*A. Deproteinizing Solution* Modified Abraham's reagent

*1. Deaminating Reagent* Nineteen parts of borac acid fluoride mixture mixed with 1 part of bromine solution

*C. Reagents for Liberation of Iodine*

*1. Potassium iodide* 10 per cent solution

*2. Hydrochloric acid, 6N (18 per cent)* equal volumes of concentrated hydrochloric acid and distilled water

**NOTES** Potassium bromate was found most convenient for making up bromine solution B<sub>4</sub>, as suggested by Rappaport and Eichhorn. The deaminating reagent was found to keep for about three hours after which it should be made up fresh. The strength of this reagent should be adjusted by varying the proportion of bromine solution B<sub>4</sub> to borac fluoride solution B<sub>3</sub> so that the blank reading lies within the most sensitive range of the photometer scale, in the range 30 to 40 per cent transmittance.

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*Procedure —*

- 1 Pipette 0.100 ml of finger tip or venous blood into 5.00 ml of Aldrich's deproteinizing fluid. Mix thoroughly and filter (or centrifuge).
- 2 Into a 25 ml volumetric flask containing about 10 ml of water, pipette exactly 3.00 ml of the clear filtrate and 5.00 ml of deammating reagent.
- 3 Wait exactly two minutes after the addition of the deammating reagent and then add 0.2 ml of 10 per cent KI and 3 ml of 18 per cent HCl.

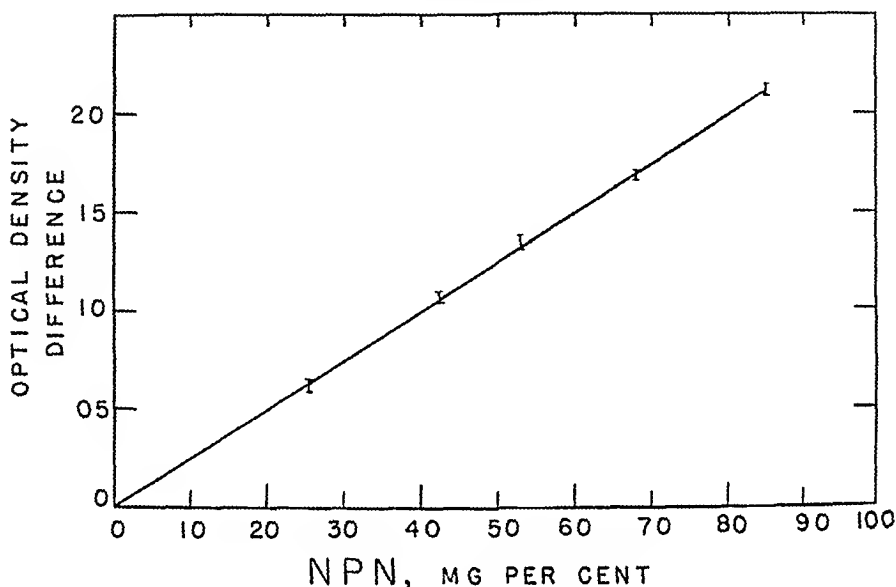


Fig. 1—Calibration curve for photometric nonprotein nitrogen estimation. The vertical axis represents differences between optical density of blank and of unknown. Coleman Junior spectrophotometer at 475 millimicrons.

- 4 Dilute to the mark with distilled water, invert to mix, and read at once in the photometer.
- 5 Prepare a reagent blank, substituting 3 ml of deproteinizing solution for the blood filtrate in (2), and proceed as described.
- 6 Subtract the optical density reading of the blood filtrate from that of the reagent blank, and, using the difference, read off the concentration of nonprotein nitrogen from the calibration curve.

**NOTES.** In procedure (2) a tube calibrated at 25 ml, such as the Folin Mahros blood sugar tube, may be substituted for the volumetric flask, a Folin Wu sugar tube is not suitable.

(3) In adding the HCl, the technician must avoid transferring KI from the mouth of the flask or tube back into the HCl stock bottle on the tip of the pipette. This will turn the acid yellow.

(4) Filter photometers such as the Lumetron and Fisher have been used, with the blue filters supplied. We used a Coleman Junior spectrophotometer, with 19 mm cuvettes set at 475 millimicrons. The wave length setting is not critical, as free iodine does not give a sharp absorption band.

(5) As the calibration curve is not linear above 100 µg per cent, blood filtrates giving a value above this must be diluted as necessary, and the determination repeated.

*Calibration*—A calibration curve was obtained by using a series of blood specimens with nonprotein nitrogen values up to 100 mg per cent. Readings were made upon these as described and the actual nonprotein nitrogen was accurately determined by a micro Kjeldahl procedure. For the various specimens, the difference in optical density between blank and blood filtrate was plotted against the nonprotein nitrogen of the whole blood in milligrams per 100 milliliters.

The calibration curve obtained with the Coleman Junior Spectrophotometer is illustrated in Fig. 1. The precision of the method is indicated by the results of replicate determinations upon separate filtrates of the same blood, as presented in Table I.

TABLE I. PHOTOMETRIC DETERMINATION OF NONPROTEIN NITROGEN IN WHOLE BLOOD (Optical density readings in Coleman Junior spectrophotometer at 415 m $\mu$ )

NPN BY MICRO KJELDAHL (MG PPT CENT)	BLANK READING	READINGS OF REPLICATE BLOOD FILTRATES	DIFFERENCE	MEAN DIFFERENCE + NPN
25.5	0.469	0.403 0.406 0.403 0.406 0.40	0.059 0.062 0.065 0.062 0.063	0.00243
45.5	0.468	0.364 0.360 0.358 0.358 0.360	0.104 0.108 0.110 0.110 0.108	0.00254
53.0	0.468	0.336 0.330 0.331 0.334 0.334	0.132 0.138 0.131 0.134 0.134	0.00253
68	0.468	0.301 0.302 0.298 0.297 0.301	0.167 0.166 0.170 0.171 0.167	0.00247
80	0.468	0.257 0.259 0.258 0.258 0.259	0.115 0.209 0.210 0.210 0.209	0.00248
Constant by method of least squares				0.00249

If a reliable micro Kjeldahl method is not available the procedure may be calibrated with a standard urea solution although urea gives readings about 5 per cent higher than blood filtrates with equivalent nitrogen contents. Weigh out accurately 200 mg. of chemically pure urea and dissolve in distilled water to 100 ml. giving a solution with nonprotein nitrogen concentration of 93.3 mg per 100 milliliters. Using this solution carry out the steps of the procedure as with whole blood. Prepare a calibration curve by making serial dilutions of this standard urea solution.

## CONCLUSION

The hypobromite method for nonprotein nitrogen in whole blood has been modified in that the liberated iodine is estimated photometrically, rather than by titration. The method is standardized by micro-Kjeldahl. It affords readily reproducible results, and is much more rapid and convenient than the conventional Folin-Wu determination.

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## STANDARDIZED REAGENT FOR THYMOL TURBIDITY TEST

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THE thymol turbidity of MacLagan<sup>1, 2</sup> has been widely accepted as an hepatic test, however, the normal range recorded by different investigators varies.<sup>1, 8</sup> The thymol reagent is a supersaturated solution. This causes a variation of the thymol concentration in different reagents prepared. This is significant since the reagent becomes turbid after a few days of standing at room temperature. In the following, the error resulting from the use of different thymol reagents is evaluated and then thymol concentration is determined. In addition, the preparation of a uniform thymol reagent is described which is not subject to the mentioned error.

### METHODS

The thymol turbidity was determined following the original description,<sup>1, 2</sup> the calibration being done with barium sulfate according to Shank and Hongland.<sup>9</sup> The units recorded with this calibration are twice as high as those with the standard of MacLagan, since the barium sulfate suspension is wrongly based on a 0.962 normal instead of molar barium chloride solution.<sup>10</sup> Inasmuch as the values can be converted into each other by simple multiplication or division by two the units are easily comparable if reference is made to the standard used.

For determination of the thymol concentration in thymol reagents, the following method was adapted.

#### *Reagents—*

- 1 Folin and Ciocalteu phenol reagent.<sup>11</sup>
- 2 2.5N NaOH
- 3 Thymol standard solution containing 60 mg of pure thymol per liter

#### *Standard Curve—*

Exactly 1, 2, 3, 4 and 5 ml of the standard solution are transferred to 50 ml volumetric flasks. Another flask is reserved for the blank. Water is added to all flasks to make approximately a total volume of 25 milliliters. Then 4 ml of 2.5N NaOH solution, and slowly, while shaking 3 ml of the Folin Ciocalteu reagent are added. The volume is made up to the mark with water and the readings are made after fifteen minutes, using a wave length of 620 millimicrons. The blue color is stable for at least twenty five minutes. The curve follows Beer Lambert's law.

#### *Results—*

*For Determination of the Thymol Concentration in MacLagan's Reagent* The reagent was diluted 5 ml to 100 ml and from this dilution 5 ml were pipetted into a 50 ml volumetric flask and the color was developed as in the standard curve simultaneously with a blank and 5 ml of the standard solution. In twenty seven thymol reagents prepared according to MacLagan on the same or different days, the thymol concentration varied from 89.5 to 117

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mg per 100 ml of reagent. The mean of the thymol concentration was 102 mg per 100 ml with a standard deviation of  $\pm 2$ . Therefore, in the following experiments a concentration of 100 mg per 100 ml of reagent was selected.

The influence of the thymol concentration of the reagent upon turbidity readings was determined by measuring the turbidity resulting from using seven different MacLagan reagents of varying thymol concentration for four sera. In general, the turbidity was proportional to the concentration of the thymol in the reagent (Fig 1). In addition, the thymol reagent with the highest thymol concentration was diluted with the barbital buffer to produce concentrations of 95, 90 and 85 per cent of the reagent. The turbidities were proportionate to the thymol concentration.

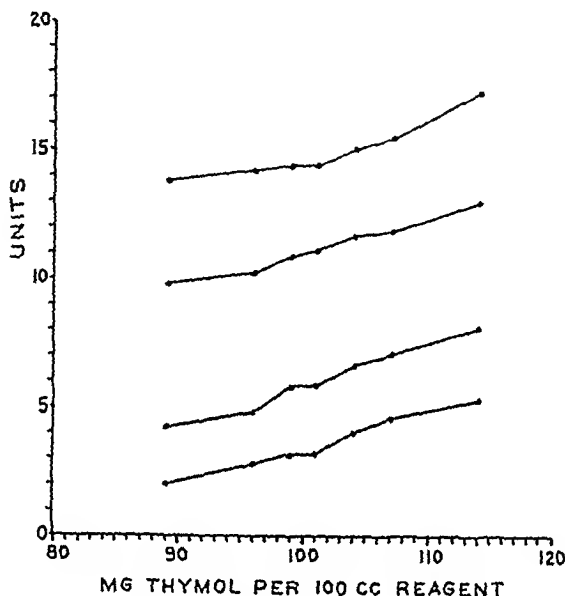


Fig 1—Relation between the thymol turbidity of four sera and the thymol concentration of the reagents

*A Thymol Reagent With Constant Thymol Concentration Was Prepared as Follows*  
Because USP thymol may contain impurities which are not soluble in ethyl alcohol, a bulk amount of thymol was dissolved in 95 per cent ethyl alcohol, and the insoluble material was removed by filtration. Cold water was added to the filtrate to precipitate the thymol. The crystals were dried on a desiccator under anhydrous calcium chloride for two or three days. Then, an exactly 10 per cent solution of the dried thymol in 95 per cent alcohol was prepared which serves as permanent stock solution. The thymol reagent is prepared by transferring about 80 ml of the barbital buffer (prepared according to MacLagan<sup>2</sup> but without thymol) to a 100 ml glass stoppered volumetric flask, and adding exactly 10 ml of the 10 per cent alcoholic thymol solution. The thin oily layer of thymol is dissolved by shaking the flask vigorously for thirty to sixty seconds and then more buffer is added to make to the mark. No oily droplets must be seen in the neck of the flask. In eighteen different lots prepared from three different stock solutions on different days, the concentration of thymol was 100 mg  $\pm$  1. If necessary, a large volume of reagent may be prepared accordingly.

Since in barbital buffers without thymol an amorphous precipitate may eventually form, probably because of the growth of mold or bacteria, a stock barbital buffer solution with half concentration of thymol which does not permit bacterial growth and which is not super saturated was prepared as follows. About 900 ml of the barbital buffer solution were transferred to a 1 liter volumetric flask, and 50 ml of the 10 per cent alcoholic thymol



solution were added. The flask was then shaken as before and filled with buffer to the mark. To prepare the thymol reagent 0.5 ml of the 10 per cent thymol stock solution is diluted as described to 100 ml with the buffer thymol solution. The reagents prepared by either method can be kept at room temperature and used until turbidity develops.

To ascertain whether the presence of 1 ml of ethyl alcohol per 100 ml interferes with the thymol turbidity test to different aliquots of Maelagan's thymol reagent 9.5 per cent ethyl alcohol was added to produce an alcohol concentration of 1, 2, and 3 per cent respectively. The turbidity readings of different sera by use of the reagent with 1 and 2 per cent alcohol did not differ from those obtained with the original reagent, while in a few sera a slight aberration was noticed with the 3 per cent alcohol reagent.

Using the alcoholic thymol stock solution a thymol reagent with thymol concentration of 120 mg per 100 ml was prepared which is about 20 per cent more concentrated than most of Maelagan's reagent. As expected with this reagent the turbidity readings of normal and pathologic sera were higher than with the original reagent. However no additional diagnostic benefit was observed from using this modification.

#### DISCUSSION

The thymol turbidity readings depend upon the thymol concentration of the reagent used. Since the latter varies in view of the slight supersaturation of the reagent it was not surprising that different lots produced different readings with the same serum. Alcohol increases the solubility of thymol in water and thus permits the preparation of uniformly supersaturated thymol reagents. Since the thymol reagent is not stable for more than two weeks and has to be prepared repeatedly it appears advantageous to use the presented modification by using a stable alcoholic stock solution and a stable buffer with half concentration of thymol which will give a uniform thymol concentration in any batch prepared.

In addition to the necessary standardization of the calibration (which takes into account the double unitage reading if the published standard of Shank and Hoagland<sup>1</sup> is used) standardization of the thymol reagent should assist in producing uniform results in the same laboratory and in different institutions.

#### SUMMARY

A modification in the preparation of the thymol reagent in Maelagan's thymol turbidity test is proposed by using a stable 10 per cent alcoholic thymol stock solution and a stable barbitol buffer with one half thymol concentration. Using this modified reagent the variations in the results due to different thymol concentrations in various batches of the original thymol reagent of Maelagan's are avoided.

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## VACUUM SAMPLING TUBE FOR RESPIRATORY GASES

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RAPID repetitive sampling of respiratory gases is often necessary and in our own case it has been needed for estimation of cardiac output. For accurate estimations a tube has been devised which will in quick succession wash out the instrumental dead space and collect the sample. The tube was designed for use

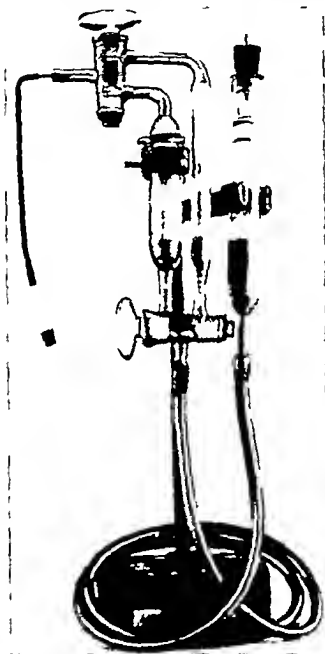


Fig. 1

with the Scholander gas analysis apparatus and has been found very suitable, for the sample can be transferred directly without the necessity of using the conventional transfer pipette thus eliminating a possible source of contamination. By increasing the dimensions of the tube it is suitable for use with the Haldane gas analysis apparatus.

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Received for publication March 11, 1949.

*Construction* —The tube is made of glass and consists of two barrels, one of half-inch internal diameter and 10 c.c. capacity to receive the sample and the other of quarter-inch internal diameter and 5 c.c. capacity to receive the dead-space air from the supply lines. A twoway cock connects the lower end of either barrel to a length of Tygon tubing (superior to rubber since it does not leak under pressure) which leads to the mercury reservoir. Another cock at the upper end connects either barrel with the intake, which is fitted with a short length of stainless steel tubing, to which may be fitted the small-bore polyvinyl tubing leading to the point from which it is desired to sample. A piece of similar tubing, six inches long with conventional rubber tip fixed at the end of a short piece of narrow-bore glass tubing, is used for transferring the sample to the Scholander apparatus. The sampling tube, with its fellows, is supported in a stand rather like a large test tube rack and is nine inches high.

*Mode of Use* —Before use, each barrel is evacuated separately in the ordinary way, and the volume of the smaller barrel evacuated can be varied with the length of line to be cleared. Sampling is then carried out when desired by turning the upper cock through a complete rotation.

The vacuum sampling tube described has been devised for operations in which rapidly obtained uncontaminated samples of gas are required, it also provides a simplified and accurate method of transfer of gases to the Scholander gas analysis apparatus.

The tube was made up in the Department of Physiology, University of Pennsylvania, by Mr J. D. Graham.

# THE HEMATOLOGIC CHANGES INDUCED IN GUINEA PIGS BY THE PROLONGED ADMINISTRATION OF PTEROYL GLUTAMIC ACID ANTAGONISTS

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ANTAGONISTS of pteroyl glutamic acid (folic acid) are currently being evaluated as therapeutic agents for the treatment of leukemia and are being used experimentally in attempts to study the physiologic relationship between pteroyl glutamic acid (PGA) and the antipermeicous anemia principle of liver. Detailed information about the hematologic effects produced by PGA antagonists is needed as a background for these observations. The experiments reported in this paper define the hematologic changes induced in guinea pigs by the prolonged administration of four different antagonists. An earlier report described the effects of relatively large doses of 4-amino pteroyl glutamic acid (4-amino PGA) and emphasized the striking difference in the susceptibility of different species of animals to the drug.<sup>1</sup> In the present study, small doses of the various antagonists were used in order that chronic rather than acute changes might be induced. Guinea pigs were selected because they occupy an intermediate position with respect to species susceptibility, and because the cytology of their peripheral blood and bone marrow is remarkably similar to that of the human being.

## I. MATERIALS AND METHODS

Except where otherwise specified adult guinea pigs weighing about 500 grams were used. Since the effects of PGA antagonists *per se* were being studied no attempt was made to influence the amount of PGA available to the animals either through diet or intestinal synthesis. Lettuce and Purina Rabbit Chow were fed throughout the experiments. Antagonists were administered by subcutaneous injection at intervals of twenty-four hours.

Before injection of an antagonist was started each animal was observed during a control period of two to five days to establish its normal range of weight and its peripheral blood values. Throughout the time of drug administration, frequent determinations were made of the red blood cell count, hemoglobin value, reticulocyte percentage, total and differential white blood cell counts and the platelet level. For the reticulocyte and platelet estimations, Dameshek's platelet solution was used.<sup>2</sup> Hemoglobin was estimated by the Evelyn method.<sup>3</sup>

Bone marrow was aspirated from the iliac crest of guinea pigs in quantities sufficient to allow the preparation of satisfactory marrow films. A 20 gauge spinal puncture needle, complete with stylet, was cut to a length of one and one-half inches. The animals were firmly held, face down, with the hind legs fully extended after shaving and cleansing the skin over the ilium, a small cut was made in the tough skin and the sterile needle introduced.

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Commonwealth Fund Fellow in Medicine.

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TABLE I THE PERIPHERAL BLOOD OF NORMAL GUINEA PIGS

AUTHOR	NUMBER OF ESTIMATIONS	MEAN FBC (MILLIONS/ C MM) WITH RANGE	MEAN HB WITH RANGE	MEAN RETICULO CYTE PERCENTAGE WITH RANGE	MEAN WBC/ C MM WITH RANGE	MEAN PLATELETS (MILLIONS/ C MM)	MEAN DIFFERENTIAL WBC COUNT WITH RANGE				
							T %	L %	M %	F %	B %
Scarborough <sup>14</sup>	529*	5.75 (4.5-6.8)	90-100 % (S-11th)	1.4			41.8	45.3	8.4		
	829*				10,700 (6,000-20,000)		41.8	45.3	8.4	4.8	0.77
Wintrobe <sup>5</sup> King and Lucase	4	5.75 5.06 (4.7-5.25)	14.5 Gm %		17,300 (13,000-23,000)						
	9 guinea pigs followed for 18-21 days						31.1 (0.76)	63.1 (20.97)	1.8 (0.10)	3.5 (0.14)	0.2 (0.2)
Present investigation	48	5.63 (4.4-6.7)	15.2 Gm % (13.6-17.9)	1.2 (0.1-3.0)	10,600 (5,000-18,700)	1.7	44.7 (20.76)	47.6 (21.74)	5.2 (1.12)	1.2 (0.4)	1.3 (0.5)

\*Figures collected from the literature

through the muscle until contact was made with the crest of the bone. A boring movement allowed the tip of the needle to enter the marrow cavity, so that on withdrawal of the stylette a small quantity of marrow blood and flecks could be aspirated into a 5 cc syringe. The flecks were spread on slides and stained with Wright's stain. In about 75 per cent of aspirations attempted in this manner enough marrow was obtained for satisfactory examination. The operation appeared to cause little lasting discomfort to the animals and although it was not felt desirable to repeat the procedure on the same side until at least ten days had elapsed, aspiration from the opposite ilium was carried out on the next day in some animals where rapid changes in the marrow cytology were anticipated.

No guinea pigs were sacrificed during the investigation, though several died from the effects of the drugs. Autopsy was then performed as soon as possible and the weight and gross appearances of the organs were noted. Fixed sections of liver, spleen, kidney, suprarenal heart, and lung were made for microscopic examination.

(a) *The Peripheral Blood and Bone Marrow of Normal Guinea Pigs*—Normal values for the cellular elements in the peripheral blood of guinea pigs are summarized in Table I. The tabulation includes data obtained by other workers as well as those from the present investigation.

The marrow in healthy guinea pigs is cytologically similar to normal human marrow (Fig. 1). It has been described in detail by Epstein and Tompkins<sup>7</sup> figures given by them for the differential counts agree essentially with those found for the normal animals in the present study. Instead of reporting results in terms of differential counts however, it was deemed to describe the alterations induced by the PGA antagonists. This was done for two reasons: (1) careful descriptions of the appearance and distribution of the cell types probably convey a more accurate impression of any cytologic changes, and (2) in the treated animals, primitive cells were often encountered which were difficult to classify with conviction.

(b) *The Pteroyl Glutamic Acid Antagonists Used*—The chemical names of the four antagonists employed in this investigation and the symbols which will be used to identify them are tabulated below.

NAME	SYMBOL
Pteroyl aspartic acid—racemic form	An Fol R†
Pteroyl aspartic acid—active form	An Fol A†
7 methyl pterotic acid	Met Fol B†
4 amino pteroyl glutamic acid	Aminopterin 4 amino PGA

Each of the first three antagonists was given alone to some of the animals and in combination with 4 amino PGA to others.

## II. RESULTS

A *Pteroyl Aspartic Acid—R*—This compound was given to four guinea pigs. One animal (Guinea pig 1) received a daily dose of 20 mg, another (Guinea pig 3) 10 mg, and the other two (Guinea pigs 2 and 4) 5 mg each. These dosages were continued for seventy four, seventy two, sixty two, and fifty three days respectively. No general ill effects were noted throughout the experiment; the animals maintained their weight, appetite, and normal activity.

In all four animals a mild prolonged reticulocytosis, ranging from 4 to 13 per cent, developed after about ten days and continued for twenty to thirty days. In three animals (Guinea pigs 1, 3, and 4) this reticulocytosis was pre-

<sup>7</sup>Supplied by the Lederle Laboratories Division of the American Cyanamid Company to whom the authors express their appreciation.

†Names used by the Lederle Laboratories to identify these compounds.

ceded by a fall in the erythrocytes to approximately 4 million per cubic millimeter, followed by a spontaneous return to normal levels. Otherwise, no changes

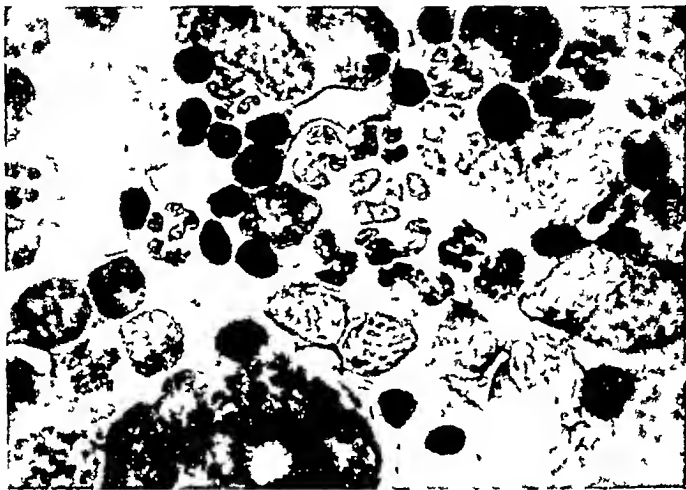


Fig 1—Marrow of a normal guinea pig (X850)

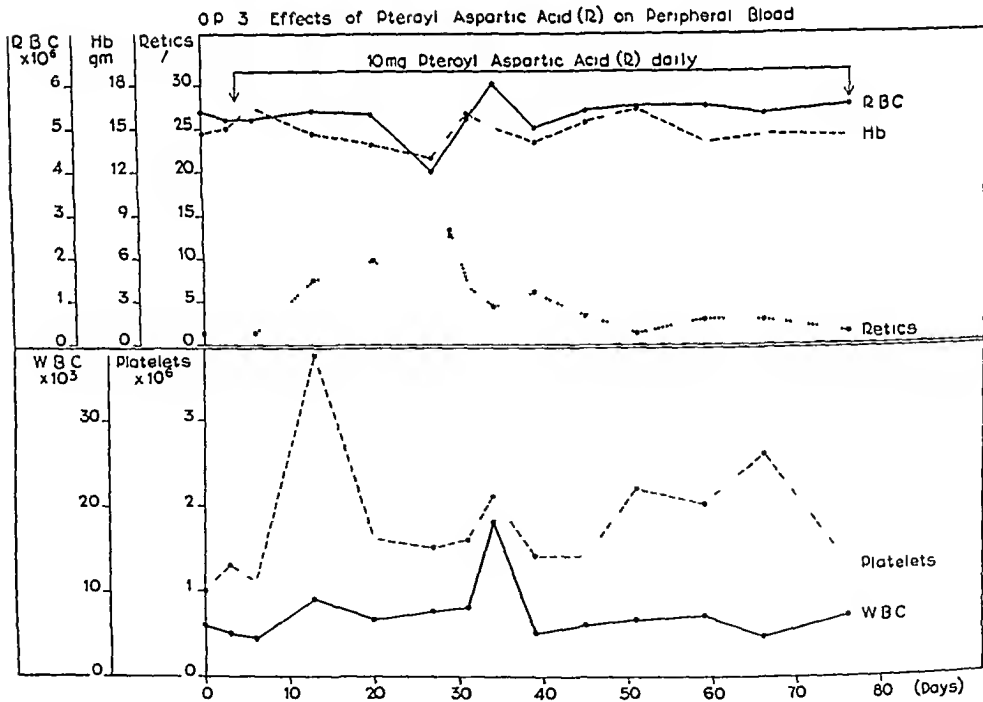


Fig 2

were noted in the peripheral blood of any of the animals, the white blood cell, differential and platelet counts remained within normal limits throughout. Hematologic data for Guinea pig 3 are graphically recorded in Fig 2



Bone marrow aspiration was successful in three of these animals (Guinea pigs 1, 2 and 4) at the completion of the experiment. Apart from a slight increase in the orthochromatic and polychromatic normoblasts, no morphologic change was observed.

*B Pteroyl Aspartic Acid—A*—This optically active form of pteroyl aspartic acid was administered to one animal (Guinea pig 5) in daily dosage of 20 mg for sixty seven days. No general ill effects were observed and the only hematologic change observed in the peripheral blood was an intermittent polymorphonuclear leucocytosis rising to levels as high as 28,000 cells per cubic millimeter. Marrow aspiration at the end of the experiment showed no abnormality other than an increase in metamyelocytes, band forms and polymorphonuclear neutrophils.

*C 7 Methyl Pteric Acid*—Two animals (Guinea pigs 6 and 7) were given daily doses of 5 mg and 10 mg of this drug respectively. After forty days the dosage was increased to 10 mg and 20 mg daily and continued for an additional twenty two days. No general ill effects were noted.

On the lower dosage both pigs showed a mild reticulocytosis, ranging from 5 to 8 per cent, which appeared after seven days and continued for approximately twenty five days. The red corpuscles remained within normal limits during the experiment. The total leucocyte count in both animals rose within ten days from control levels of 10,000 to 15,000 per cubic millimeter to around 20,000 per cubic millimeter and remained thus while the drug was being given. This slight increase affected both the granular and nongranular forms. The platelet values showed no striking change.

Marrow aspiration was unsuccessful in these animals.

*D 4 Amino Pteroyl Glutamic Acid*—4 amino PGA was given to eight animals (Guinea pigs 8 to 15) in six of which (Guinea pigs 8 to 13) the effects of prolonged daily administration were observed.

Three animals (Guinea pigs 8 to 10) received 0.25 mg daily for ninety two, sixty five, and sixty three days respectively. Guinea pigs 9 and 10 maintained their weight and appetite throughout. In Guinea pig 9 the red blood count had fallen gradually to 3.5 million per cubic millimeter after sixty five days, but otherwise neither of these two animals showed any hematologic changes. Guinea pig 8 maintained its weight for sixty four days on the 0.25 mg dose but thereafter lost weight steadily and died on the ninety second day. During this whole period the red blood cells remained essentially normal. About the fiftieth day a leucocytosis of 23,000 cells per cubic millimeter developed because of an increase in both neutrophils and lymphocytes. The elevated white blood cell count continued until the ninetyeth day though after the eightieth day it was accounted for entirely by a granulocyte increase, the lymphocytes falling sharply at this time. In the terminal two days a dramatic fall occurred in the total white blood cells (from 24,250 to 2,350 cells per cubic millimeter) and in the platelets (Fig 3).

Marrow studies were made in the latter animal eight days before death and post mortem. At the time of the peripheral leucocytosis, the marrow showed a marked increase in metamyelocytes, band and segmented neutrophils (Fig

4,A), although the red blood count at this time was normal, there was a definite increase in pronormoblasts and basophilic normoblasts. Megakaryocytes of normal appearance were seen. There was also present a number of large cells, of irregular outline with nongranular, pale blue-staining cytoplasm and large nuclei with an open chromatin network and occasional nucleoli. For reasons to be discussed later, these cells were considered to be "reticulum cells."

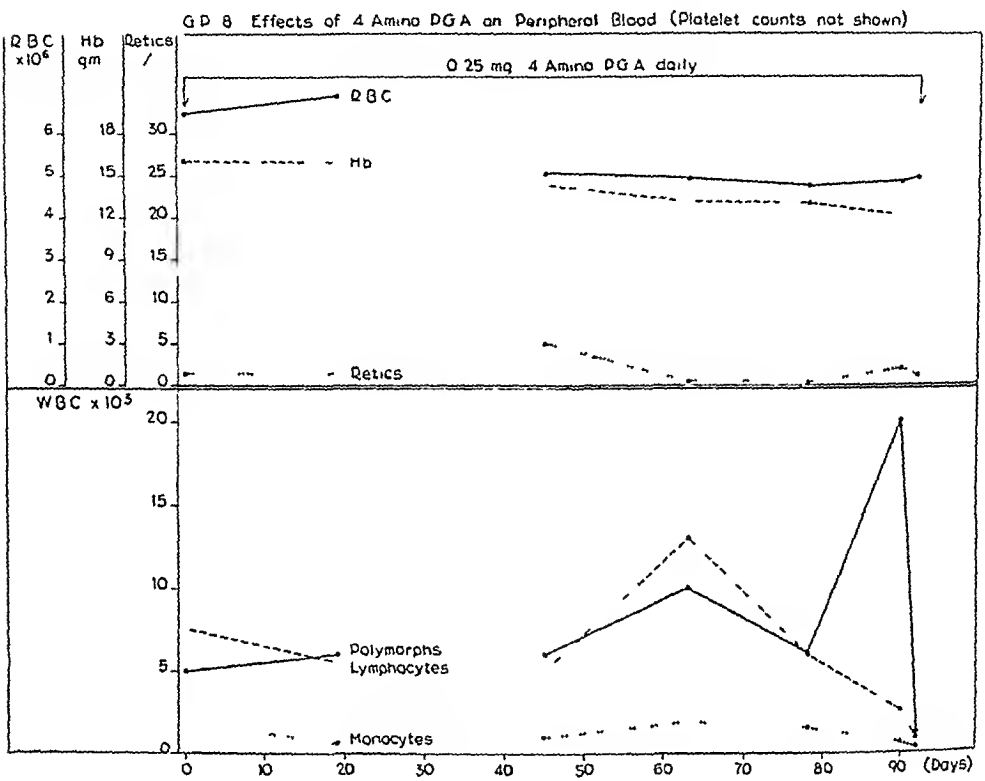


Fig. 3

In the post-mortem marrow of the same animal (Guinea pig 8, Fig 4,B), myelocytes were still present in considerable numbers but there was an almost total absence of metamyelocytes and polymorphonuclear granulocytes. The number of reticulum cells had definitely increased, their wide distribution and the general cellularity of the marrow could best be appreciated in fixed section (Fig 4,C)

No abnormalities of the viscera were noted grossly at autopsy and no hemorrhages were observed macroscopically. On microscopic examination the liver showed extreme fatty change affecting the central and mid-zonal cells of the lobules (Fig 4,D). An area of recent hemorrhage was seen in the medulla of one suprarenal. In the splenic pulp the endothelial elements were prominent, but apart from these findings the organs showed no changes.

Three animals (Guinea pigs 11, 12, and 13) were given prolonged daily dosage of 0.5 mg 4-amino PGA.

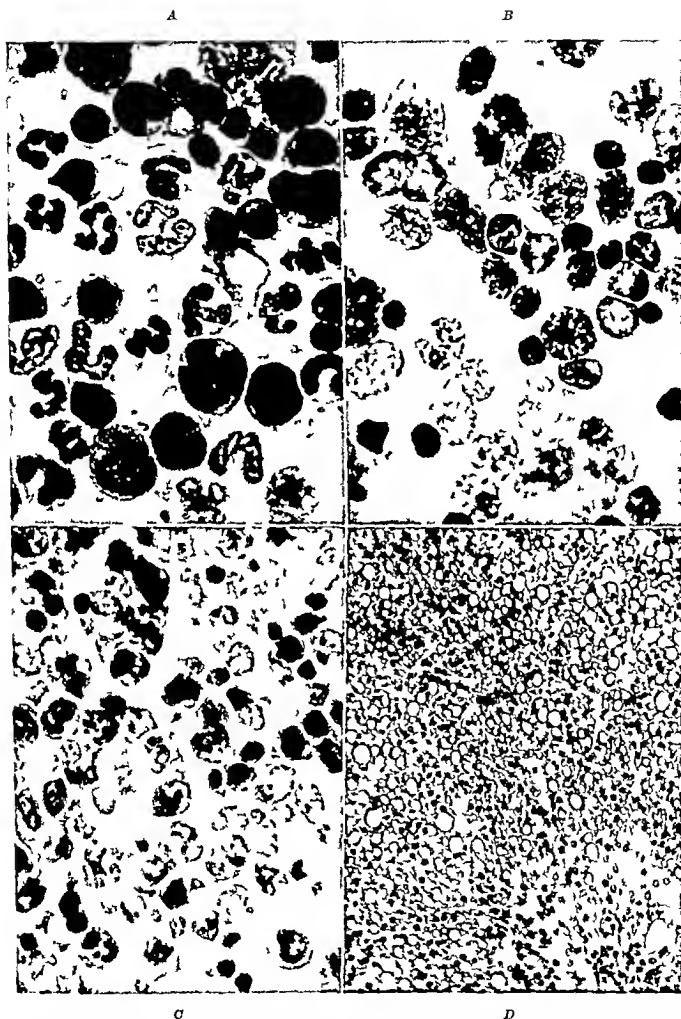


Fig 4.—Culnea pig 8. *A* Bone marrow during peripheral leucocytosis ( $\times 80$ ). *B* Bone marrow film after terminal leucopenia (post mortem  $\times 80$ ). *C* Bone marrow section after terminal leucopenia (post mortem  $\times 80$ ). *D* Liver ( $\times 10$ ).

The first of these lost weight and died in thirty-two days. During this time its red blood cell count fell to 3.7 million per cubic millimeter and its leucocytes to 3,250 per cubic millimeter, the differential count showed a reduction in both granulocytes and lymphocytes. There was also a terminal drop in platelets. At the time of death, the marrow was very cellular and contained a large number of reticulum cells. The red cell precursors were decreased, and in the granulocytic series there was an almost complete absence of segmented and band forms. Myelocytes, however, were numerous, with basophile and eosinophile forms especially prominent. Megakaryocytes were scanty. Microscopic study of tissue sections showed areas of recent hemorrhage and edema in the lungs and an increase in the endothelial cells in the spleen. No fatty change was observed in the liver, and other organs examined showed no significant abnormalities.

The second animal (Guinea pig 12) was given 0.5 mg 4-amino PGA for forty-eight days, at the end of which time it appeared to have suffered no general ill effects. No changes were observed in the peripheral blood other than a very gradual decline in the erythrocyte level to 4.22 million per cubic millimeter.

The third animal (Guinea pig 13) received the daily 0.5 mg dosage for 144 days with no apparent change in its general condition. In the peripheral blood, a fall in erythrocytes to 3.03 million per cubic millimeter in thirty-six days was partly corrected by a subsequent reticulocytosis which continued for thirty days and attained a maximum of 15 per cent. Thereafter, the red blood cell level remained between 3.5 million and 4.0 million per cubic millimeter until the one hundred sixteenth day. While administration of 4-amino PGA was continued, 10 mg PGA were then injected daily for fourteen days, followed by 5 USP units of liver extract daily for an additional two weeks. These supplements caused no increase in reticulocytes, and the red blood cell count fell gradually to 2.9 million per cubic millimeter. Up to this time (one hundred forty-fourth day) no change had occurred in the total or differential leucocyte count or in the number of platelets. Marrow aspiration studies were made on the one hundred sixteenth day and one hundred forty-fourth day, i.e. before and after the PGA and liver extract. On the one hundred sixteenth day the marrow was very cellular and showed erythroid hyperplasia with a large number of pronormoblasts and basophilic and polychromatic normoblasts. The granulocytic precursors and megakaryocytes appeared normal. A considerable number of reticulum cells was present. On the one hundred forty-fourth day, the marrow cytology was essentially unchanged (Fig 5, A and B).

In two animals (Guinea pigs 14 and 15) the effects of giving a large single dose of 4-amino PGA were investigated.

Guinea pig 14, weighing 510 grams, received an injection of 20 mg of the compound. No changes were observed in its general condition or in its peripheral blood during the succeeding fourteen days, aspirated marrow examined on the tenth day following the injection showed no abnormality. A second injection of 20 mg produced the same negative result. Doses of 2 mg were then administered daily for ten days, the white blood cell count fell to 5,500 per cubic millimeter but there was little change in the red blood cell and hemoglobin levels. The dosage was next reduced to 1 mg daily for thirty-two days.

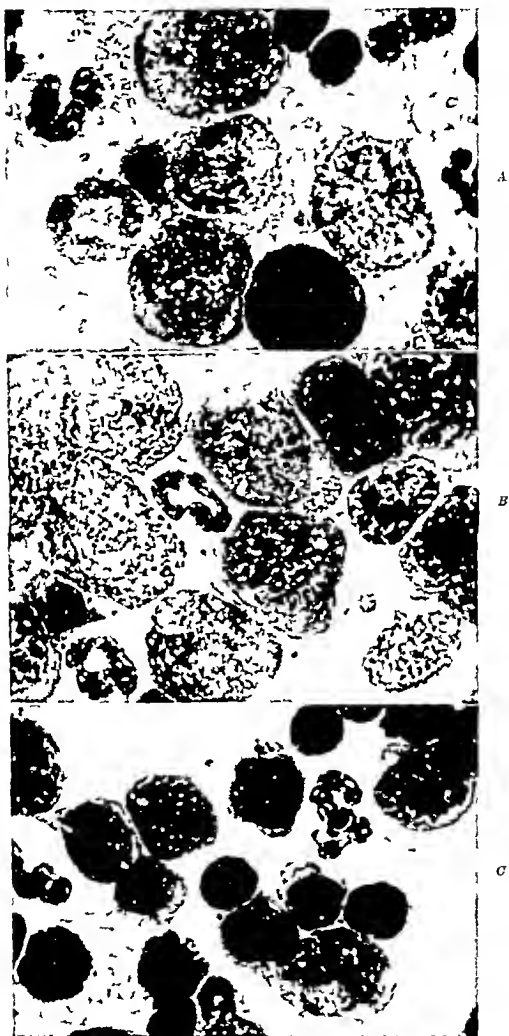


Fig 5—Guinea pig 13. A Bone marrow after 0.5 mg 4-amino PGA had been given daily for 116 days ( $\times 1400$ ). B Bone marrow after PGA and liver extract one hundred forty fourth day ( $\times 1400$ ). C Bone marrow five days after stopping 4-amino PGA ( $\times 1400$ ).

During this time, the red blood cells decreased to 2.68 million per cubic millimeter and the hemoglobin level fell to 7.8 Gm per cent, no further depression occurred in the leucocytes, and the differential and platelet counts remained normal. Marrow examination at the end of the period showed an increase in

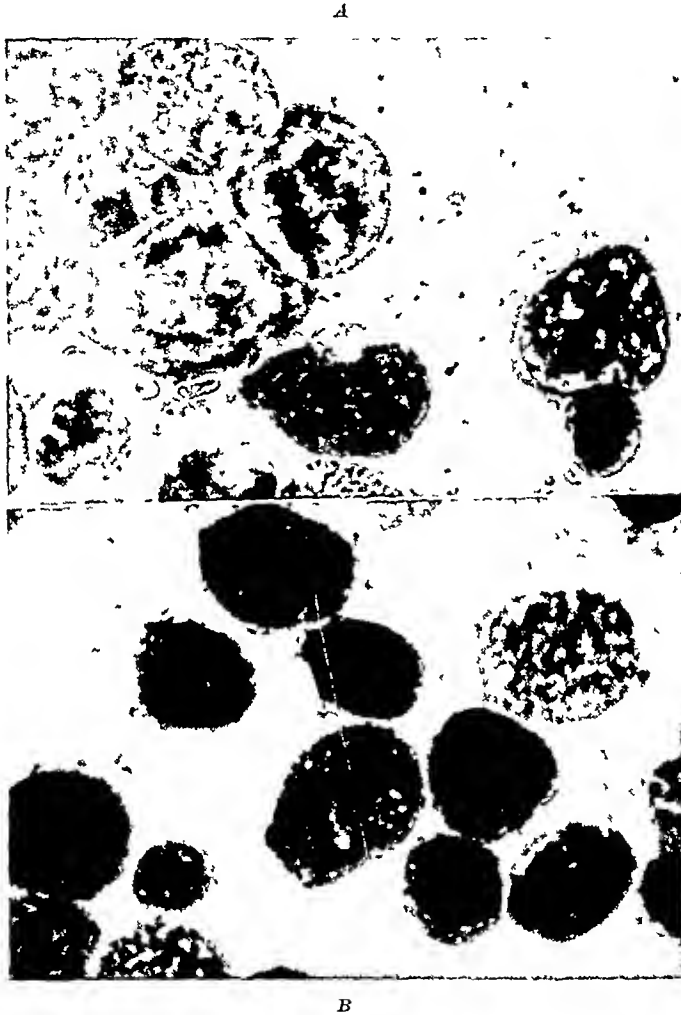


Fig 6—Guinea pig 14. A Bone marrow before stopping 4-amino PGA ( $\times 1400$ ) B Bone marrow twenty-four hours after stopping 4-amino PGA ( $\times 1400$ )

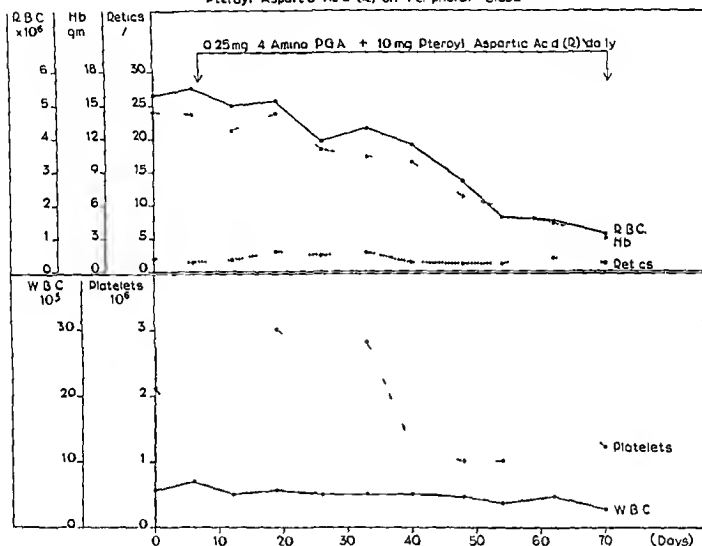
the number of pronormoblasts and basophilic and polychromatic normoblasts, with no change in the white cell precursors or megakaryocytes. Reticulum cells and plasma cells were prominent (Fig 6,A).

The second animal (Guinea pig 15, 830 grams) was given a single injection of 40 mg of 4-amino PGA. Observations over the next fourteen days revealed no general or hematologic effects. Doses of 4 mg daily were then given for ten days, and while no changes were found in the peripheral blood, the marrow at the end of this time showed a decrease in the proportion of erythroid precursors with pronormoblasts and basophilic normoblasts outnumbering the later forms

In the granulocytic series all stages of precursors were present, though the proportion of early myelocytes was increased. Reticulum cells, plasma cells, and lymphocytes were numerous.

*E 4 Amino PGA and Pteroyl Aspartic Acid—R*—The combined effects of these two antagonists were studied in five animals (Guinea pigs 16 to 20). Table II indicates the daily dosage of each drug, the duration of administration,

OP 16 Effects of Combined Doses of 4 Amino PGA and Pteroyl Aspartic Acid (R) on Peripheral Blood



Fig

and the findings in the peripheral blood before and after the experiments. In all cases a general depression of erythrocytes, leucocytes, and platelets resulted. A severe anemia developed in animals 16 and 19 and marked leucopenia in Guinea pigs 16 and 18. In both of the latter differential counts showed an almost equal reduction in granulocytes and lymphocytes. Weight loss occurred in Guinea pigs 16 and 18 though animal 16 was the only animal in the series to die (Fig 7).

Marrow examined at the end of dosage in Guinea pigs 16, 17, 19, and 20 was cellular in each instance. There was an increase in promyeloblasts and basophilic and polychromatic normoblasts with a diminution in orthochromatic normoblasts. In the anemic Guinea pig 16 the total erythroid elements were reduced with promyeloblasts as the predominating type. The granulocytic

TABLE II EFFECTS ON THE PERIPHERAL BLOOD OF COMBINED DOSAGE WITH AMINOPTERIN AND AN FOL R

	DAILY DOSE OF AMINOPTERIN (MG)	DAILY DOSE OF AN FOL R (MG)	DURATION OF DOSAGE (DAYS)	R B C (MILLIONS/C MM)		H B (GM %)		W B C /C MM		PLATELETS (MILLIONS/C MM)
				BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	
QUINIA PIG										
16	0.25	10	64	5.47	1.09	14.2	3.1	6,700	2,450	1.35
17	0.25	10	57	5.34	4.23*	14.0	11.1	7,500	4,950	1.75
18	0.25	5	48	6.43	4.73	14.8	12.2	6,800	1,900	0.52
19	0.25	20	25	4.68	4.69	14.9	13.4	10,100	11,400	3.25
	0.5	20	38	4.69	2.24	13.4	6.4	11,600	8,300	1.30
20	0.25	5	109	5.67	4.31†	15.1	11.7	16,500	12,300	3.0
	0.25	10	25	4.31	3.55	11.7	10.1	12,600	10,500	1.10

\*R B C fell to 3.31 millions/cmm but rose again to 4.23 millions/cmm following spontaneous reticulocytosis reaching 20.5 per cent

†R B C fell to 3.62 millions/cmm but rose again to 4.31 millions/cmm following spontaneous reticulocytosis reaching 20.6 per cent

TABLE III EFFECTS ON THE PERIPHERAL BLOOD OF COMBINED DOSAGE WITH AMINOPTERIN AND MET FOL B

	DAILY DOSE OF AMINOPTERIN (MG)	DAILY DOSE OF MET FOL B (MG)	DURATION OF DOSAGE (DAYS)	R B C (MILLIONS/C MM)		H B (GM %)		W B C /C MM		PLATELETS (MILLIONS/C MM)
				BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	
GUINEA PIG										
23	0.25	10	42	5.27	4.13	15.4	11.4	15,300	200	0.14
24	0.25	20	65	5.61	1.93	16.3	6.4	19,700	3,950	0.4
25	0.25	20	20	6.08	3.97	16.7	10.4	12,100	1,400	0.1



precursors appeared normal except for a marked decrease in polymorphonuclear leucocytes and band forms in Guinea pig 16. All four marrows showed megakaryocytes of normal appearance. Reticulum cells were numerous in every instance.

Post mortem examination in animal 16 showed a large, pale liver weighing 24 grams and splenic enlargement to about three times the normal size. There were no signs of hemorrhage. Microscopically there was a marked degree of fatty change in the liver with vacuolation of the central and mid zonal cells of the lobules. Endothelial elements in the spleen were prominent. Other organs showed no significant changes.

*F 1 Amino PGA and Pteroyl Aspartic Acid—Active Form*—Two animals (Guinea pigs 21 and 22) were given daily injections of 0.25 mg of 4 amino PGA and 20 mg An Fol A.

Both lost weight, Guinea pig 21 died after twenty four days and Guinea pig 22, at the end of forty six days. Peripheral blood examinations showed a gradual decline in erythrocytes and in hemoglobin though at the time of death neither animal was severely anemic (3.67 million and 3.39 million per cubic millimeter). The white blood cell count for Guinea pig 21 fell from an initial value of 18,000 per cubic millimeter to 7,500 two days before death; the absolute number of lymphocytes decreased from 15,000 to 600 per cubic millimeter. Unfortunately, no terminal white blood count was obtained. Guinea pig 22 developed a leucopenia (1,450 per cubic millimeter) before death; the differential count showing a parallel fall in neutrophils and lymphocytes. Platelets remained virtually unchanged.

Marrow examination at the time of death showed in both guinea pigs a decided increase in the number of pronormoblasts and early myelocytes. The more mature granulocytic precursors were considerably reduced and megakaryocytes were present though scanty. Reticulum cells were numerous; plasma cells and lymphocytes were found in more than normal numbers.

Necropsy revealed no macroscopic signs of hemorrhage in either animal. The liver was large in Guinea pig 21 (weight 23 grams), but showed no histologic abnormality. There was increased endothelial activity in the splenic pulp of Guinea pig 22. No other abnormalities were observed in either animal.

*G 1 Amino PGA and 7 Methyl Pteric Acid*—These two antagonists were administered simultaneously to three animals (Guinea pigs 23, 24 and 25). The amounts, duration and effects on the peripheral blood are shown in Table III.

All three animals died. Guinea pigs 23 and 25 showing marked loss of weight. Leucopenia and thrombocytopenia developed terminally and there was some depression of erythrocytes in each pig; the anemia in Guinea pig 24 was severe. Changes in the leucocytes followed a general pattern: first there was an increase in the neutrophils and monocytes with no change in the lymphocytes; then a sharp fall occurred in all three white cell types (Fig. 8).

In Guinea pigs 23 and 25 the bone marrow became uniformly hypoplastic for erythrocytic granulocytic and megakaryocytic elements; the most prominent cells were reticulum cells, plasma cells, and lymphocytes. Of the erythroid and

myeloid series, the majority were early forms pronormoblasts and young myelocytes. The marrow of the severely anemic animal (Guinea pig 24) was actively cellular and showed an increase in the pronormoblasts and basophilic normoblasts with relatively few more mature forms. Considerable numbers of very early myelocytes were seen together with bands and segmented neutrophils,

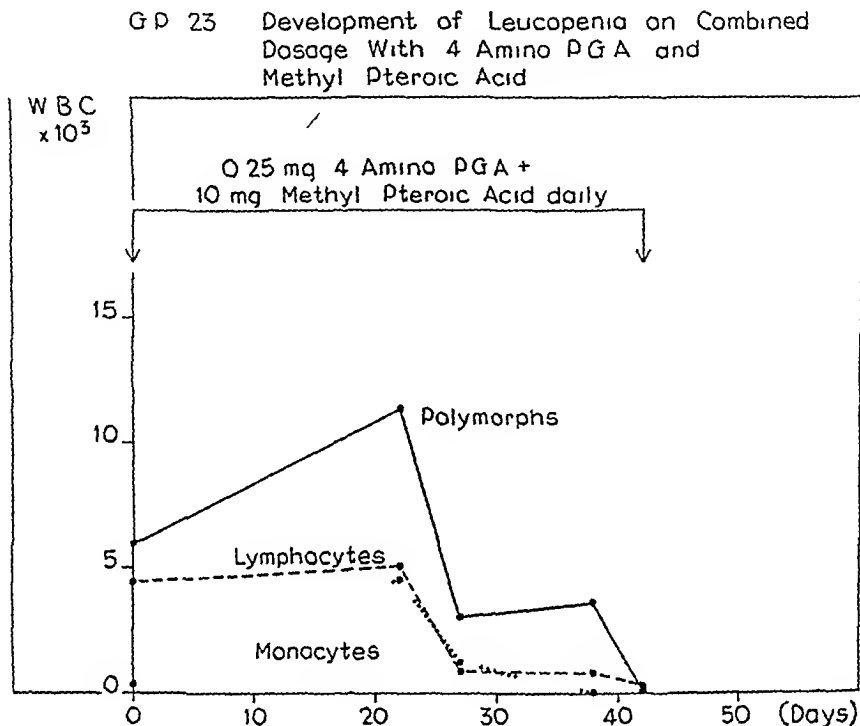


Fig 8

but the intermediate myelocyte and metamyelocyte stages were largely missing. Megakaryocytes were of normal appearance. In this marrow also, reticulum and plasma cells were numerous.

Macroscopic and microscopic examination of the tissues of these three animals showed little of note except for endothelial proliferation in the splenic pulp and a moderate degree of fatty change toward the center of the liver lobules in Guinea pigs 24 and 25.

*II Hematologic Recovery From Effects of Antagonists*—The peripheral blood and marrow changes which occurred when administration of the antagonist compounds was discontinued were observed in six of the animals in Guinea pigs 13 and 14 after 4-amino PGA alone, and in Guinea pigs 17, 18, 19, and 20 after combined dosage with 4-amino PGA and An-Fol-R.

Hematologic recovery occurred with striking rapidity in each instance (Table IV). Figs 9 and 10 show the detailed course of recovery from anemia and leucopenia in Guinea pigs 14 and 18 respectively. In every animal a reticulocytosis was evident within forty-eight hours after the last injection and reached a maximum (amounting to over 40 per cent in Guinea pigs 14 and 19)

GP 14 Spontaneous Recovery From Anemia After Stopping Dosage With Folic Acid Antagonist

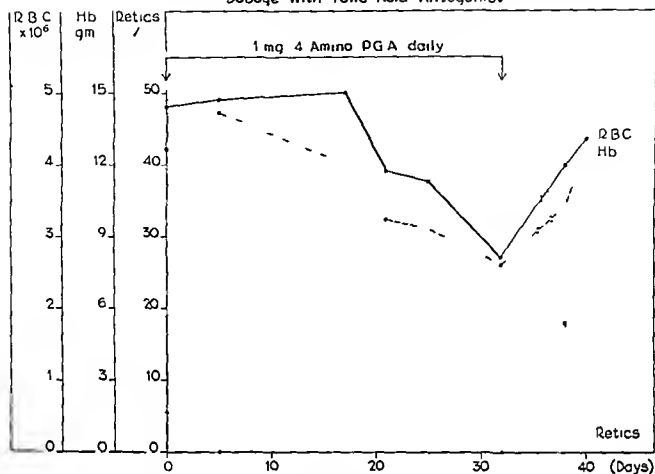


Fig 9

OP 18 Spontaneous Recovery From Leucopenia After Stopping Dosage With Folic Acid Antagonists

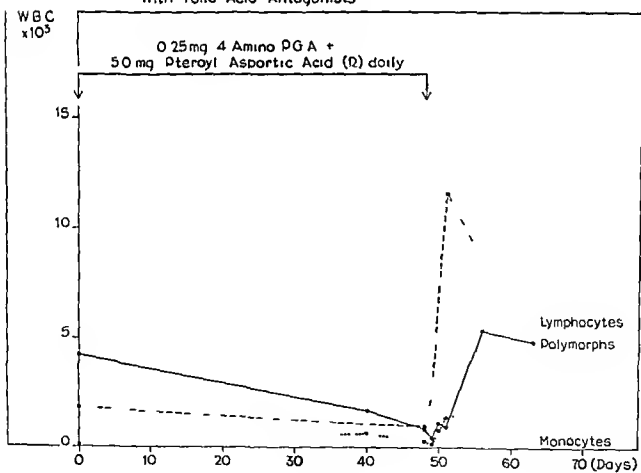


Fig 10

TABLE IV PERIPHERAL BLOOD FINDINGS BEFORE AND AFTER STOPPING DOSAGE OF FOLIC ACID ANTAGONISTS (FIGURES IN PARENTHESES INDICATE NUMBER OF DAYS FOLLOWING CESSATION OF DRUGS)

GUINEA PIG	RBC (MILLIONS/C MM)		HB (GM %)		RETICULOCYTE PERCENTAGE		WBC/C MM		PLATELETS (MILLIONS/C MM)	
	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
13	2.9	6.33 (12)	10.7	14.6 (12)	0.2	12.4 (3)	8,600	18,400 (19)	1.45	1.95 (12)
14	2.68	4.35 (8)	7.8	12.7 (8)	0.0	40.8 (4)	8,700	9,650 (10)	1.0	0.75 (8)
17	4.23	4.72 (10)	11.1	13.4 (10)	1.0	15.5 (3)	4,950	11,550 (8)	1.75	3.25 (3)
18	4.73	5.87 (8)	12.2	15.2 (15)	1.7	6.2 (4)	1,900	16,250 (8)	0.52	1.5 (8)
19	2.24	5.10 (6)	6.4	11.8 (10)	0.1	43.1 (5)	8,300	16,700 (3)	1.30	1.85 (3)
20	3.55	4.83 (10)	10.1	13.0 (10)	0.1	14.1 (3)	10,450	18,300 (10)	1.10	2.40 (10)

within three to five days. There was a rapid return of the red blood cell count toward normal, with a noticeable lag in the rate of hemoglobin recovery in some instances. In all the animals, cessation of the drug was followed also by a rise in leucocytes. Lymphocytes were the first cells to increase in number in every instance (Fig 10).

Marrow was studied in three animals at different stages of the recovery phase. In Guinea pig 14, marrow was obtained twenty-four hours after the last dose of 4-amino PGA. Compared with the marrow of the previous day, there were definite signs of increased maturity of the erythrocytic precursors, with polychromatic and orthochromatic normoblasts more numerous although large numbers of pronormoblasts still remained (Fig 5, B and C). A complete return to normal erythropoiesis was evident in the marrow of Guinea pig 19 obtained ten days after the last administration of 4-amino PGA and An-Fol-R.

### III DISCUSSION

The purpose of this discussion is to examine the experimental results obtained and to determine whether they indicate the mode of action of the PGA antagonists.

*4-Amino PGA*—Of the four compounds employed in this investigation, 4-amino PGA was obviously the most active hematologically. The smallest dose (0.25 mg daily) produced recognizable effects only when given over a very long period (nine to thirteen weeks), but when 0.5 mg was administered daily, marrow and blood changes appeared much earlier.

In the bone marrow the effects of 4-amino PGA were characterized by an increasing number of early erythrocytic and/or granulocytic precursors. The general cellularity tended to be increased rather than decreased. In all instances the change toward immaturity was accompanied by the appearance of a great many large, primitive-looking cells with pale, nongranular cytoplasm and large nuclei with an open chromatin meshwork. The cytoplasm of these cells was sometimes vacuolated, their nuclei usually showed nucleoli and were not infrequently divided. These cells were seen only occasionally in normal guinea

pig marrow. It was decided that they were most probably reticulum cells since their morphologic characteristics corresponded with the description of the latter given by Jaffe<sup>8</sup> and other workers. The earlier red blood cell precursors were studied with a view to ascertaining whether dosage with a PGA antagonist would produce a maturation arrest of the type seen in pernicious anemia. Whereas large numbers of pronormoblasts were found, no cells were seen which possessed all of the cytologic features of the megakaryoblasts of pernicious anemia.

The changes in the peripheral blood reflected those in the bone marrow. In general, when the erythroid elements in the marrow were retarded in maturation, a slowly developing anemia became evident. Repeated examination of blood films indicated this anemia to be normocytic and normochromic in type. In two instances when the red cells appeared macrocytic, determination of absolute cellular constants yielded normal values. Leucopenia in the peripheral blood accompanied a diminution in the more mature granulocytic precursors in the marrow. Thrombocytopenia occurred as a terminal event in two animals, in both of which evidence of tissue hemorrhage was found post mortem. Although the marrow of these latter pigs contained a few megakaryocytes, it seemed possible that some of the large double nucleated reticulum cells, also present in considerable number, might represent very early forms of platelet precursors.

In the animals which developed leucopenia, there was a marked diminution in the number of circulating lymphocytes as well as neutrophils. This finding, together with the histologic evidence of proliferation of the endothelial elements in the splenic pulp, suggests that 4-amino PGA may exert a general effect on reticuloendothelial tissue. The advanced degree of fatty change in the liver cells observed in one of the animals which died from the effects of the drug suggests that it also may have a systemic toxic action.

It was found in two animals (Guinea pigs 14 and 15) that a large single dose of as much as 20 to 40 mg. could be administered without producing any hematologic abnormality in the peripheral blood. Moreover, in animals in which prolonged daily dosage had brought about a marked retardation of hematopoietic output, signs of a return toward normal blood formation were evident in the marrow within twenty-four hours after administration was stopped. These two results suggest that in guinea pigs 4-amino PGA is excreted rapidly and that its action is dependent upon the maintenance of a sufficient concentration in the body tissues. Furthermore, unless the animals were moribund, hematologic effects produced by the drug appeared to be completely reversible merely by stopping dosage. In one animal a previous attempt to reverse the changes by the administration of PGA and liver extract was unsuccessful.

*An Fol R, An Fol A, and 7 Methyl Pteric acid*—These three compounds were all given over prolonged periods in doses as large as 20 mg. daily, with remarkably little hematologic effect. Apart from a mild continued reticulocytosis in all instances and a persistent elevation of the leucocyte count in the two animals given 7-methyl pteric acid and the one given An Fol A, no general or hematologic changes were observed.

*Combined Dosage With 4-Amino PGA and Other Antagonists*—In the series of animals in which the effects of combined dosage were investigated, the amount of 4-amino PGA given was chosen as 0.25 mg daily, a dose which had been shown previously to produce minimal changes over a long period. To this were added the same amounts of An-Fol-R, An-Fol-A, and 7-methyl pterioic acid which had appeared relatively inactive when given alone. The object was to determine whether these three compounds would have any potentiating effect on the action of 4-amino PGA.

4-amino PGA and An-Fol-R, given simultaneously to five animals, produced anemia and leucopenia in all of them, with one death. 4-amino PGA and An-Fol-A caused a moderate degree of anemia and leucopenia in two animals, both of which died. 4-amino PGA and 7-methyl pterioic acid produced anemia with profound leucopenia and terminal thrombocytopenia, killing all three animals observed. In general, these changes occurred in a distinctly shorter period than was required to produce demonstrable effects by the use of 4-amino PGA alone, but the resulting marrow changes were in every way similar to those observed with the latter drug. While this series of experiments is too small to allow definite conclusions to be drawn, it does suggest that An-Fol-R, An-Fol-A, and 7-methyl pterioic acid are themselves true, though weak, depressants of hematopoiesis.

The nature of this investigation does not permit speculation as to the biochemical action of the PGA antagonist compounds. However, on the basis of the cytologic effects, it seems permissible to consider briefly the mechanism whereby the hematologic changes were brought about. The actions of the four antagonists studied are probably similar, though 4-amino PGA is by far the most powerful agent.

The action in guinea pigs would seem to be a generalized slowing of maturation of all blood cell precursors found in normal marrow. The effects may be manifested mainly on one or more of the series of precursors and tend to result in the production of an ever-increasing number of early forms and reticulum cells. Thus, while the marrow becomes more and more filled with primitive cells, anemia, leucopenia, or thrombocytopenia may develop in the peripheral blood before a terminal pancytopenia occurs. These changes are quickly and spontaneously reversible unless dosage with the drugs is maintained.

#### IV. SUMMARY

1 The hematologic effects of prolonged administration of pteroyl aspartic acid (R and A), 7-methyl pterioic acid, and 4-amino pteroyl glutamic acid to a group of twenty-five guinea pigs are described in detail.

2 4-amino PGA was found to be the most active compound in depressing hematopoiesis with the production of immature cell types in the bone marrow, and anemia and/or leucopenia in the peripheral blood.

3 The other antagonists studied produced little hematologic effect when administered alone, but each appeared capable of potentiating the action of minimal dosage of 4-amino PGA when given simultaneously.

4 The marrow changes produced were found to be rapidly and spontaneously reversible unless dosage with the compounds was maintained

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# THE EFFECTS OF NITROGEN MUSTARD ON INDUCED ERYTHROBLASTIC HYPERPLASIA IN RABBITS

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## INTRODUCTION

THE successful therapy of various benign or neoplastic disease processes with ionizing radiation or chemicals depends to some extent on the selective sensitivity of certain offending cell types. Occasionally, as in the treatment of Hodgkin's disease with the nitrogen mustards, no unusual sensitivity of the Hodgkin's tissue appears to obtain as compared with the normal hematopoietic marrow<sup>1</sup>. It would appear that a significant clinical effect is related to a comparable destruction or depression of normal and neoplastic hematopoietic processes but with a more rapid recovery in the former. This problem of selective and sustained depression of one cell type as opposed to others in the hematopoietic system is well demonstrated in polycythemia rubra vera where hematologic remissions of several years may be induced by a single dose of radiophosphorus<sup>2</sup> or a single course of nitrogen mustards<sup>3</sup>. A serious limitation in the use of a drug such as the nitrogen mustards (methyl-*bis*-( $\beta$ -chloroethyl) amine hydrochloride) is related to the fact that in some instances the neoplastic process recurs more rapidly than the recovery of the normal hematopoietic tissue, thus making further adequate therapy dangerous or impossible<sup>4</sup>. Likewise the failure of the skin or other normal structures of the body to recover rapidly enough if at all from the absorption of ionizing radiation primarily directed to underlying or adjacent neoplasm imposes the same restriction. These remarks only serve to indicate the importance of determining ways and means of rendering normal cells of the body temporarily more resistant to the therapeutic agent than the cell population of the neoplasm. It was reported recently by Jacobson and associates<sup>5, 6, 7</sup> that the erythropoietic tissue of the rabbit could be protected from the destruction of  $\gamma$ -radiation if the erythropoietic tissue were previously stimulated by the withdrawal of blood or the administration of phenylhydrazine. Since the biologic effects of ionizing radiations and methyl *bis*-( $\beta$ -chloroethyl) amine hydrochloride† are so similar, especially as they effect the hematopoietic system,<sup>2, 8</sup> it was decided to determine whether stimulation of erythropoiesis would similarly reduce the degree of destruction produced by the latter chemical agent. This communication relates these studies. Certain studies with  $\gamma$ -radiation carried on simultaneously are included for comparative purposes.

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†Hereafter referred to as HN<sub>2</sub> or nitrogen mustard



## MATERIALS AND METHODS

Swift's snuffle free rabbits of uniform age and weight (2½ to 3 kilograms) were used exclusively in this study. The rabbits were divided into four groups (Table I). Group 1 consisted of normal, untreated animals. Group 2 received 3 mg of nitrogen mustard intravenously per kilogram of body weight. Group 3 consisted of rabbits with a phenylhydrazine induced anemia and Group 4 received 3 mg of nitrogen mustard intravenously after an anemia had been induced by phenylhydrazine.

TABLE I. NUMBER OF ANIMALS AND TREATMENT OF VARIOUS GROUPS. HEMATOLOGIC STUDIES

GROUP	PHENYLHYDRAZINE	NITROGEN MUSTARD	NUMBER OF ANIMALS
1	-	-	17
2	-	3 mg/kg	19
3	40 mg	-	10
4	40 mg	3 mg/kg	37

In preliminary experiments it was found that 30 mg of nitrogen mustard per kilogram of body weight was approximately the LD<sub>50</sub>/14 day dose for rabbits. This means that 3 mg per kilogram were sufficient to kill 50 per cent of the rabbits within fourteen days. On the other hand 800 r of x radiation is the LD<sub>50</sub>/30 day dose in our laboratory for this strain of rabbit.<sup>9</sup> No adequate criterion exists on which to base the comparability of these totally different agents. Since the histologic damage in the marrow present at three days was more or less comparable after 3 mg per kilogram of nitrogen mustard or 800 r whole body x irradiation these doses were arbitrarily chosen as equivalent doses for the purposes of this study.

**Technique of Nitrogen Mustard Administration**—Three milligrams of nitrogen mustard per kilogram of body weight were slowly injected into the marginal ear vein of the rabbit immediately after the chemical had been dissolved in physiologic saline. A concentration of 1 mg. of the drug per 1 cc. of saline was used since this concentration produced no sloughing at the site of injection and did not obliterate the ear vein.

**Technique of X Irradiation**—The x rays administered in these experiments were generated on a 200 kVp machine operating at 15 milliamperes. A 0.5 mm. copper filter and a 1 mm. aluminum filter were used. The half value layer in copper of the filtered beam was 0.98 millimeter. The exposure was measured with a Victoreen condenser meter equipped with a 100 r chamber. Measurements were made in air within a treatment box at a position occupied by the center of the animal's body. The exposure distance was approximately 30.5 inches. Victoreen chambers of 250 r full scale readings were used as monitors.

**Hematologic Studies**—The studies on the peripheral blood taken from the rabbit, using standard techniques included the following:

- (a) Erythrocytes per cubic millimeter
- (b) Hemoglobin in grams per 100 cubic centimeters
- (c) Reticulocytes in per cent of erythrocytes
- (d) Total leucocytes per cubic millimeter
- (e) Differential leucocytes

Studies on blood obtained by cardiocentesis (venous or arterial blood) included

- (f) Platelet counts
- (g) Whole blood clotting time (Lee White)
- (h) Prothrombin determinations
- (i) Heparin tolerance test
- (j) Fibrinogen and calcium determinations

These latter studies on the effect of nitrogen mustard on coagulation of blood have been reported in detail elsewhere<sup>10</sup> and will therefore not be related here.

**Histopathologic Studies**—Histologic studies were made in order to study the blood forming tissues for the proper correlation with the peripheral blood on control animals and animals given HN, only, animals given phenylhydrazine, and animals given phenylhydrazine

followed by nitrogen mustard. Animals were sacrificed at frequent intervals the first nine days and at less frequent intervals thereafter, the tissues were fixed in Zenker formol, embedded in nitrocellulose, cut at 8 or 6  $\mu$ , and stained with hematoxylin eosin azure II (Table II).

TABLE II HISTOPATHOLOGIC STUDIES SHOWING NUMBER OF ANIMALS SACRIFICED AT VARIOUS INTERVALS

TIME AFTER NITROGEN MUSTARD INJECTIONS	CONTROL RABBITS (UNTREATED)	RABBITS GIVEN NITROGEN MUSTARD (3 MG/KG)	RABBITS TREATED WITH PHENYLHYDRAZINE (40 MG/5 C)	RABBITS TREATED WITH PHENYLHYDRAZINE AND NITROGEN MUSTARD
0 hr *	1	0	1	0
1 hr	0	1	0	0
3 hr	1	1	1	2
9 hr	0	1	1	0
12 hr	0	1	1	2
1 day	1	2	1	2
2 days	0	2	1	2
3 days	1	3	1	2
4 days	0	2	1	3
5 days	0	2	1	2
6 days	0	2	0	2
7 days	1	2	1	2
8 days	1	0	0	0
9 days	0	2	0	2
12 days	1	0	0	2
14 days	1	1	1	2
16 days	0	1	1	0
21 days	0	0	2	1
35 days	1	0	2	2

\*Two days after phenylhydrazine withdrawal

## RESULTS

*Mortality Studies*—The mortality rate of the animals in Group 4 which received nitrogen mustard and phenylhydrazine was slightly higher than that of the animals of Group 2 which received mustard alone. The survival data indicate that 3 mg of nitrogen mustard injected intravenously kills approximately 50 per cent of the animals in fourteen days. The most critical period appears to be the fourth day. However, no experiments involving large numbers of animals were undertaken to determine the LD<sub>50</sub> dose and, therefore, the 3 mg per kilogram dose can be considered only a tentative and approximate LD<sub>50</sub> value.

### *Hematologic Findings*—

*Erythrocyte Values* The erythrocyte values of the animals in Groups 3 and 4 that received phenylhydrazine were reduced to  $3.3 \times 10^6$  and  $3.5 \times 10^6$  respectively at time 0 (Fig 1). The animals in Group 3 which received phenylhydrazine alone recovered from the anemia in twelve days. The erythrocyte values of the rabbits which received nitrogen mustard after the anemia had been produced (Group 4) continued to fall, reaching a mean minimum value of  $2.8 \times 10^6$  by the third day after the mustard injection, they remained thus depressed through five days, then gradually reached normal values by the twelfth day and remained within normal limits throughout the balance of the experimental period (thirty-five days). Between the sixth and twenty-second day a decrease (circa 15 per cent) in the erythrocyte values occurred in the Group 2 rabbits which received nitrogen mustard alone.

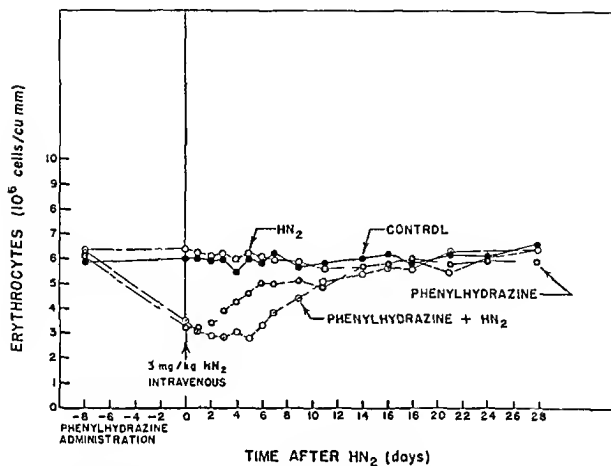


Fig 1—The effect of nitrogen mustard in a single intravenous dose of 3 mg per kilogram on the erythrocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard

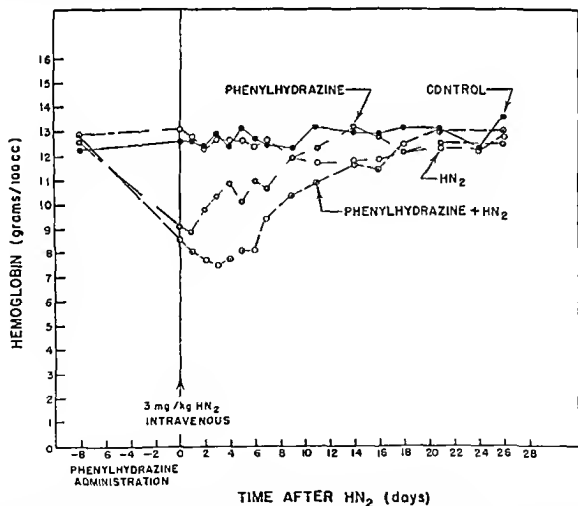


Fig 2—The effect of nitrogen mustard in a single intravenous dose of 3 mg per kilogram on the hemoglobin values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard

*Hemoglobin* The hemoglobin values (Fig 2) of the various groups followed a pattern of response comparable with that described for the erythrocytes

*Reticulocytes* The mean reticulocyte value in the control group (Group 1) varied between 3.2 and 4.2 per cent (Fig 3). The mean reticulocyte value of Groups 3 and 4 rose as a result of the phenylhydrazine administration to 22.6 and 25.4 per cent respectively. The mean reticulocyte value of Group 3 fell rapidly after the phenylhydrazine was discontinued from a maximum of 22.6 to 4.3 per cent and remained essentially in the normal range thereafter. The reticulocyte values of Group 2 which received nitrogen mustard only fell to 0.2 per cent in three days, remained below these values through the fifth day, then gradually rose to the normal range by the ninth day. The reticulocytes of the Group 4 animals, on the other hand, which received mustard after the phenylhydrazine-induced anemia fell abruptly to a minimum of 1.6 per cent by the third day, rose rapidly to 15.6 per cent by the sixth day, then gradually fell to normal by the sixteenth day.

*Leucocytes* The mean leucocyte value of the rabbits which received nitrogen mustard only (Group 2) and those that received mustard after the phenylhydrazine-induced anemia (Group 4) fell to circa 600 per cubic millimeter by the third day (Fig 4). The mean leucocyte value of the animals that received mustard alone (Group 2) continued to fall, reaching a minimum by the sixth day, after which recovery occurred by the ninth day. The leucocyte value of the animals that received nitrogen mustard after the phenylhydrazine-induced anemia (Group 4) gradually rose after the third day to an essentially normal value by the ninth day. An initial rise in the absolute heterophil value above the normal occurred in twenty-four hours in both Groups 2 and 4. In three days the heterophil value fell maximally in Group 4 animals which had received phenylhydrazine and nitrogen mustard, whereas the heterophils in the Group 2 rabbits that received mustard alone reached a minimum value in five days (Fig 5). An abortive rise occurred in both groups on the fifth and sixth days respectively. Both groups returned to normal values by the ninth day and remained essentially normal thereafter.

The absolute number of lymphocytes per cubic millimeter in Groups 2 and 4 paralleled the leucocyte curve closely (Fig 6). Partial recovery had occurred by the ninth day, but complete recovery had not occurred in thirty-five days.

The leucocyte values, including the heterophil and lymphocyte values of the animals with the phenylhydrazine-induced anemia only (Group 3), were slightly depressed by the fifth day, remained thus depressed for circa six days, rose above normal values by the sixteenth day, returned to normal values by the eighteenth day, and remained within normal limits thereafter (Figs 4, 5, and 6).

#### *Histologic Studies —*

*General Observations on Ectopic Blood Formation in Lymphatic Tissue* Scattered about the red pulp in the spleen of normal animals a few small islands

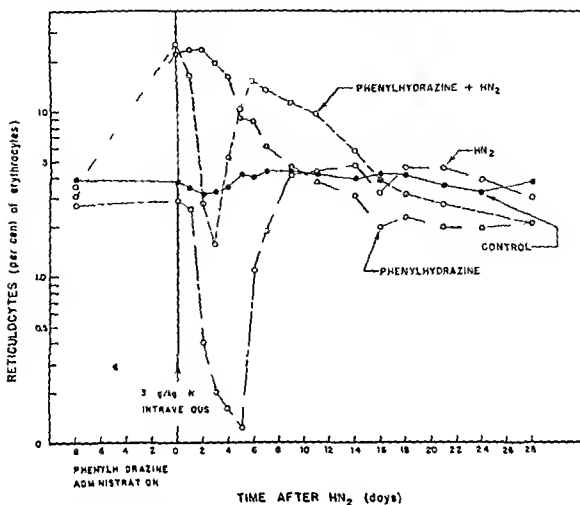


Fig. 3—The effect of nitrogen mustard in a single intravenous dose of 3 mg per kilogram on the reticulocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard

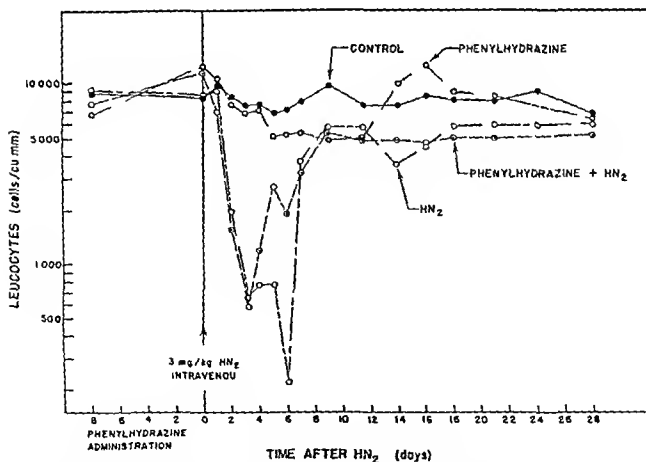


Fig. 4—The effect of nitrogen mustard in a single intravenous dose of 3 mg per kilogram on the leucocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard

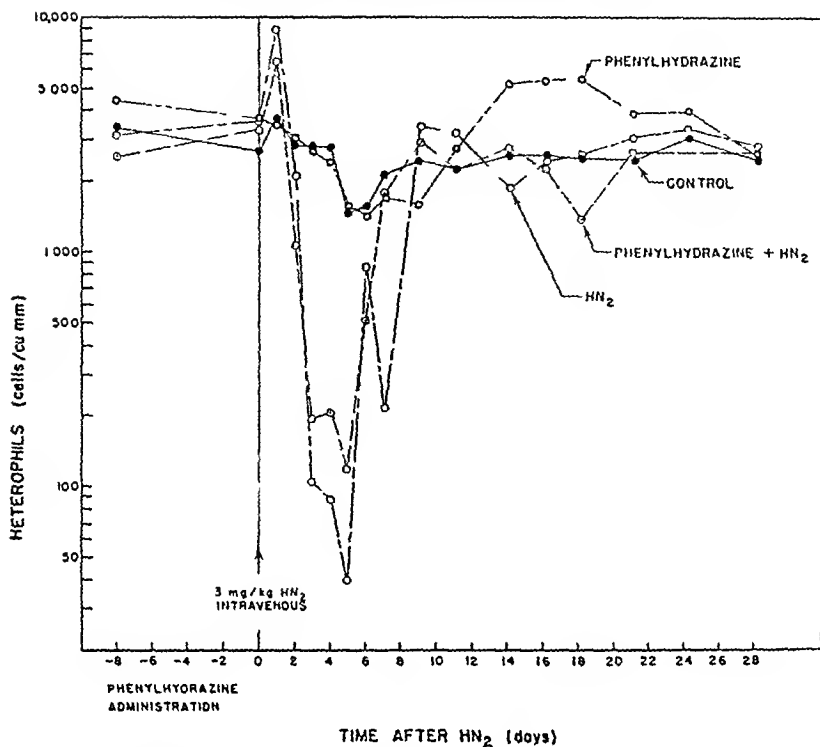


Fig 5—The effect of nitrogen mustard in a single intravenous dose of 3 mg per kilogram on the heterophil values of control rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard

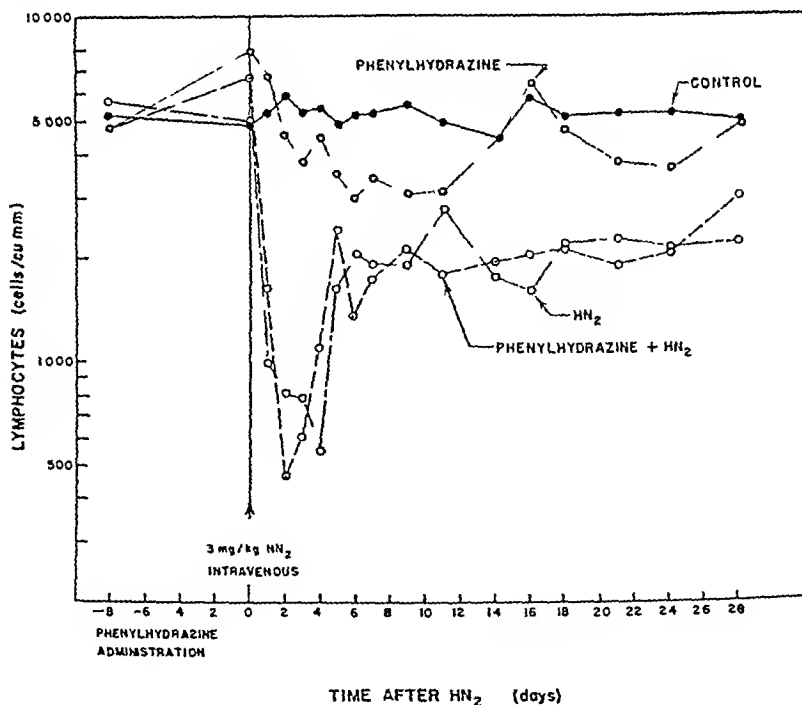


Fig 6—The effect of nitrogen mustard in a single intravenous dose of 3 mg per kilogram on the lymphocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard

of ectopic erythrocytopoiesis and granulocytopoiesis and an occasional megakaryocyte can be found. At the height of the hyperplastic reaction in the bone marrow (two days after last dose of phenylhydrazine) the amount of this ectopic blood formation in the spleen is increased by at least 100 per cent. These islands gradually decrease in extent over a period of three weeks following the last dose of phenylhydrazine leaving only the normal amount seen in most of the young adult rabbits. Ectopic blood formation in lymphatic tissue elsewhere in the body was not increased appreciably by phenylhydrazine.

*Effect of Nitrogen Mustard on Lymphatic Tissue*—The effect of HN on the dose employed on the lymphatic tissue of the thymus, mesenteric node, and spleen will only be summarized. An intense degeneration of lymphocytes was apparent within the first twenty-four hour period. In fact, six hours after injection of HN<sub>2</sub> lymphocytes were in all stages of disintegration in the lymph nodes, thymus and spleen. By the twelfth hour the degeneration phase was very marked and many macrophages within the nodules and sinuses were filled with particulate debris. Mitosis in lymphocytes was not seen at the twenty-four hour stage. Twenty-four hours after injection the debris-laden phagocytes were almost entirely gone except in the nodules of the spleen, lymph nodes and thymus, and only minimal evidence of continued cell death was evident. At the twenty-four hour stage however, there was a marked heterophil invasion of the lymph node red pulp of the spleen and of the thymus to a lesser degree. Two days after HN<sub>2</sub> injection the lymphocytes of the spleen had practically disappeared except for a few small to medium lymphocytes about the central arterioles of the nodules. The lymphocyte population of the lymph nodes and thymus, though markedly depleted, was so to a lesser degree than that of the spleen. Only a few granulocytes scattered throughout the red pulp were found in these lymphatic structures at this stage. The atrophic phase lasted through the fifth day in these lymphatic tissues. During this period little evidence of regeneration was evident. Only occasional mitoses were seen in the lymphocytes. From the sixth day stage on however the amount of lymphatic tissue in the spleen, lymph node and thymus slowly increased. Mitoses were again more prominent. Not until about the fourteenth day had the histologic reconstitution of lymphatic tissue reached the state which made differentiation from the normal control difficult if not impossible.

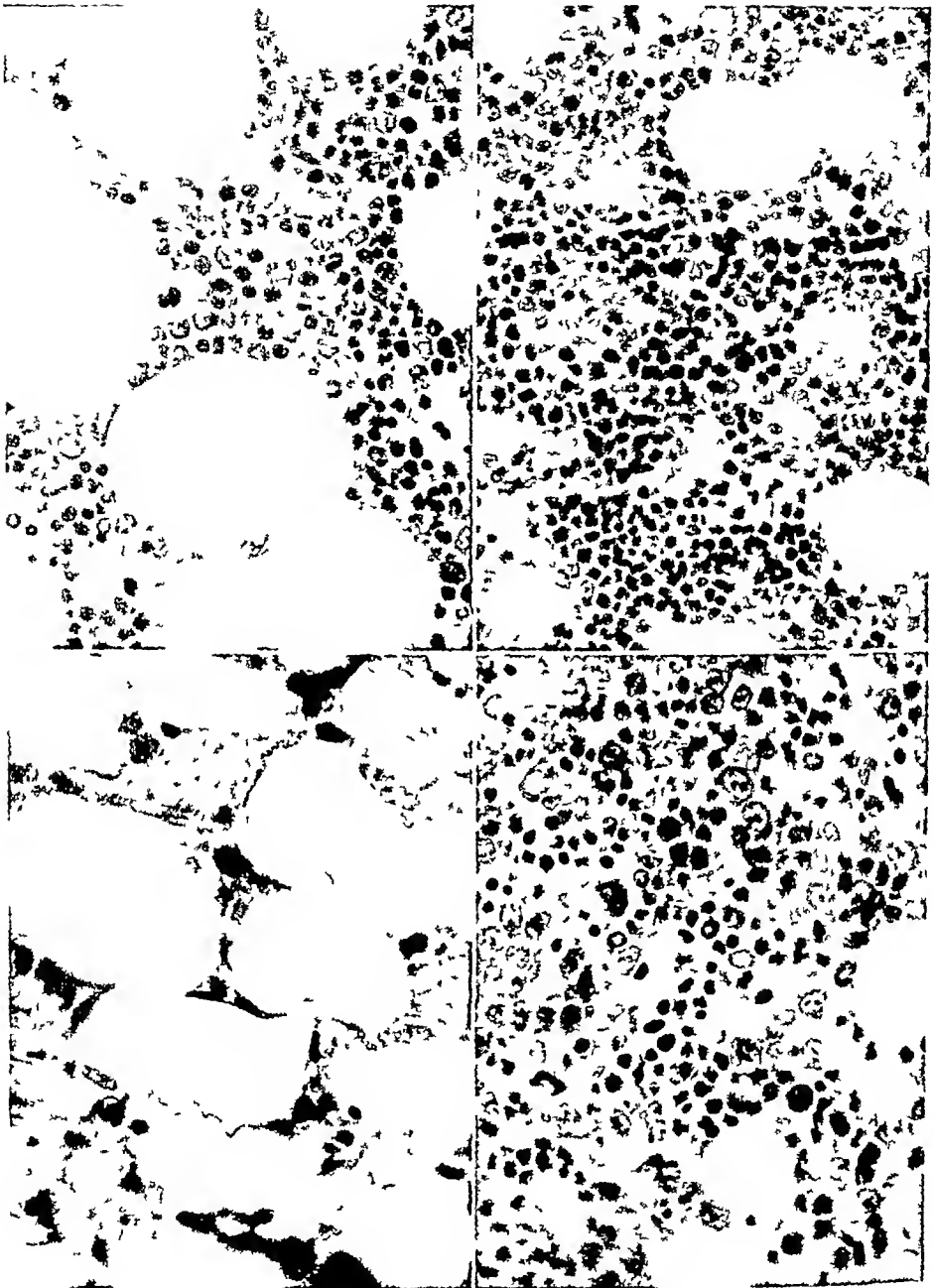
The effect of a single injection of nitrogen mustard on ectopic erythrocytopoiesis and granulocytopoiesis in the spleen was difficult to assess in terms of any differential sensitivity or insensitivity because of the small amount of such tissue present in the spleen. There was however a definite decrease in amount of ectopic erythrocytopoiesis and granulocytopoiesis following HN<sub>2</sub> injection.

*Bone Marrow*—

(a) *Femoral Bone Marrow of Normal Rabbit (Group 1) and Rabbits With Hyperplastic Marrow (Two Days After Last Phenylhydrazine Injection)* The femoral marrow of the normal control rabbits (Group 1) used in this study was composed of roughly 60 per cent fat. The balance of the marrow was composed of hematopoietic tissue. The free cells were predominantly granulo and erythrocytopoietic but contained scattered hemocytoblasts, megakaryocytes and

A

B



C

D

Fig 7—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine ( $\times 420$ )

A, Normal

B, Four days after last injection of phenylhydrazine. Note the general hyperplasia and reduction in size of fat spaces

C, Two days after intravenous injection of nitrogen mustard in a dose of 3 mg per kilo gram. The space normally occupied by marrow cells is filled with a homogeneous basophilic staining material and the general cellularity is drastically reduced

D, Two days after intravenous injection of nitrogen mustard (3 mg per kilogram) and four days after phenylhydrazine withdrawal. Note that fat spaces are small, the general cellularity is greater than normal, hemocytoblasts and basophilic erythroblasts are abundant and larger than normal



plasma cells. Only relatively few proerythroblasts (basophilic erythroblasts) were found. Mitoses were seen in granulocytes, erythrocytes, and megakaryocytes but were not prominent. In general this active hematopoietic tissue was largely concentrated in the metaphysis and along the endosteum of the entire diaphysis, diminishing toward the center of the diaphysis. The variation in the activity of the normal marrow is shown in Figs 7, 8, and 9.

The femoral marrow of rabbits two days after the last dose of phenylhydrazine (Group 3) was extremely hyperplastic. The hematopoietic marrow occupied from 85 to 95 per cent of the marrow space. Fat was correspondingly decreased. This hyperplastic marrow consisted chiefly of an increase in hemocytoblasts (Downey's myeloblast) and erythroblasts in all stages of maturation. The increase in basophilic erythroblasts was particularly conspicuous. Mitoses in these latter cells were numerous. A modest increase of granulocyte precursors and megakaryocytes was also apparent. The marrow was replete with all stages of transformation of reticular cells to erythroblasts or hemocytoblasts. The marrow hyperplasia reached its maximum two days after withdrawal of phenylhydrazine, paralleling the maximum reticulocyte response in the peripheral blood of this group (Fig. 3). Only gradually over a period of approximately two weeks did the bone marrow return to a normal state of activity after withdrawal of phenylhydrazine. Figs 7, 8, 9, and 10 illustrate the marrow hyperplasia and the lessening of hyperplasia following phenylhydrazine withdrawal. As the erythroblastic hyperplasia in the bone marrow increased the amount of phagocytized iron increased but was evident also in the later intervals after phenylhydrazine withdrawal as the marrow returned to a normal state. As indicated previously, it was at the height of the hyperplasia (two days after last injection of phenylhydrazine) that  $\text{HN}_2$  was given to the rabbits in Group 4. Animals in Group 2 (normal, previously untreated animals) were given  $\text{HN}_2$  simultaneously.

(b) *Effect of  $\text{HN}_2$  on Normal Animals (Group 2) and Animals With Hyperplastic Marrow (Group 1). Period of Degeneration (Zero to Twenty-four Hours Following  $\text{HN}_2$  Injection).* Degeneration (pyknosis, karyorrhexis, and phagocytosis) was apparent in both the granulopoietic and erythropoietic cells as early as six hours after the injection of  $\text{HN}_2$  in both Group 2 (normal animals which received  $\text{HN}_2$ ) and Group 4 (animals which received  $\text{HN}_2$  after a phenylhydrazine induced anemia), but the intensity of this degeneration was increased at the twelve hour stage. Phagocytosis of debris in the marrow was marked at the twelve hour stage. Destruction of megakaryocytes was not as obvious. Some megakaryocytes were in a stage of disintegration in twenty-four hours. The destruction of the more mature erythroblasts was more rapid than the destruction of granulocytes. In twenty-four hours the cells of the marrow of the normal rabbits which received  $\text{HN}_2$  (Group 2) were largely granulopoietic with a few scattered erythroblasts (mostly basophilic), hemocytoblasts and megakaryocytes.

The marrow of the animals whose marrow was hyperplastic when  $\text{HN}_2$  was given (Group 4) contained granulocytes, scattered megakaryocytes and numerous hemocytoblasts and basophilic erythroblasts at this twenty-four hour

stage. Again transformation of reticular cells to erythroblasts and hemocytoblasts was conspicuous. Mitoses were absent in the marrow of the animals which received  $\text{HN}_2$  only (Group 2) in six hours and none were seen at subsequent intervals studied through twenty-four hours. Mitoses were present in all the intervals studied during this destructive phase in the animals which had phenylhydrazine and  $\text{HN}_2$  (Group 4). Mitoses were largely confined to the erythroblasts. They were fewer in number than in the animals that received phenylhydrazine only (Group 3) at a comparable stage, but at least 200 per cent higher than in normal untreated controls (Group 1). Little basophilic-staining "gelatinous" marrow was evident in animals which received  $\text{HN}_2$  only (Group 2) at the twenty-four hour interval. In the phenylhydrazine and  $\text{HN}_2$  animals (Group 4), however, gelatinous marrow as well as dilated vessels were prominent, but the residual cellularity of the latter was much greater.

*Period of Atrophy (Twenty-Four Hours Through Four Days)*—The marrow of animals which received  $\text{HN}_2$  only (Group 2) was almost completely devoid of cells on the second, third, and fourth day intervals and consisted of dilated vessels, some gelatinous material, scattered reticular cells, unidentified mononuclear cells, occasional normal or giant erythroblasts, hemocytoblasts, megakaryocytes, normal or giant myelocytes, plasma cells, and fat cells. Only rarely were mitotic figures observed during this period. This extreme atrophy is illustrated in Figs 7 and 8.

The marrow of animals which received phenylhydrazine and  $\text{HN}_2$  (Group 4) contained a large amount of basophilic-staining gelatinous material, dilated vessels and phagocytized debris. Large islands of hyperplastic marrow were scattered about and were composed almost exclusively of hemocytoblasts and basophilic erythroblasts two days after  $\text{HN}_2$  injection. Other areas contained many of these precursors as well as more mature erythroblasts (Fig 7, D). The amount of residual granulopoietic and megakaryocytopoietic cells was greater than in the marrow of animals at a corresponding interval which received  $\text{HN}_2$  only (Group 2). Many giant myelocytes and erythroblasts were seen scattered about as were clover-leaf patterns in the erythroblasts. There was a questionable increase in the number of plasma cells. The surviving hemocytoblasts and basophilic erythroblasts overwhelmed the other cell types in numbers and many parts of the marrow were composed exclusively of one or the other or both. The size of these cells as compared with similar cells in the marrow of the other groups (1, 2, and 3) is illustrated in Figs 7, 8, and 9. The amount of erythrocytopoietic marrow on the second day was estimated to be at least 200 per cent greater than normal marrow (Group 1), 200 to 300 per cent greater on the third day, and 300 to 400 per cent greater on the fourth day. Mitoses were very prominent in the erythroblasts at these intervals. Granulopoietic and megakaryocytopoietic cells and the mitotic activity of these cells increased slowly at first but were relatively quite prominent by the fourth day. As was evident during the period of destruction (zero to twenty-four hours), transformation of reticular cells to erythroblasts and hemocytoblasts was prominent. In fact, direct transformation of reticular cells to giant myelocytes was becoming decidedly more prominent during the third and fourth days after  $\text{HN}_2$  in this group (Group 4).

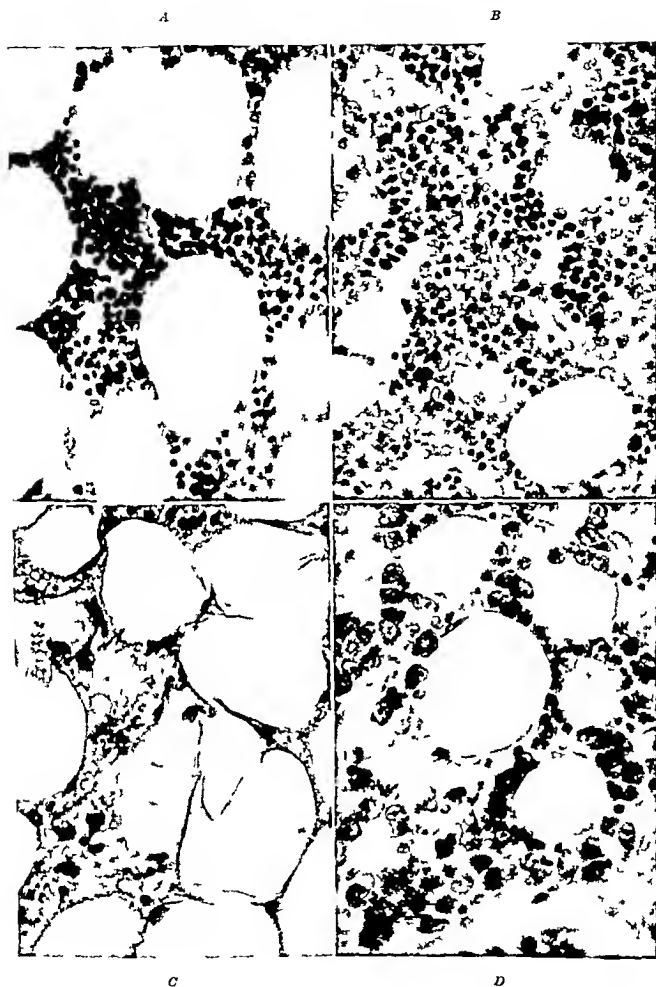


Fig 8—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine ( $\times 40$ )

A Normal

B Six days after last injection of phenylhydrazine the general hyperplasia still exists

C Four days after intravenous injection of nitrogen mustard in a dose of 3 mg per kilogram Cellular depletion marked

D Four days after intravenous injection of nitrogen mustard (3 mg per kilogram) and six days after phenylhydrazine withdrawal Cell population largely hemocytoblasts and basophilic erythroblasts Note the size of these precursors as compared with cells in normal marrow

4

B

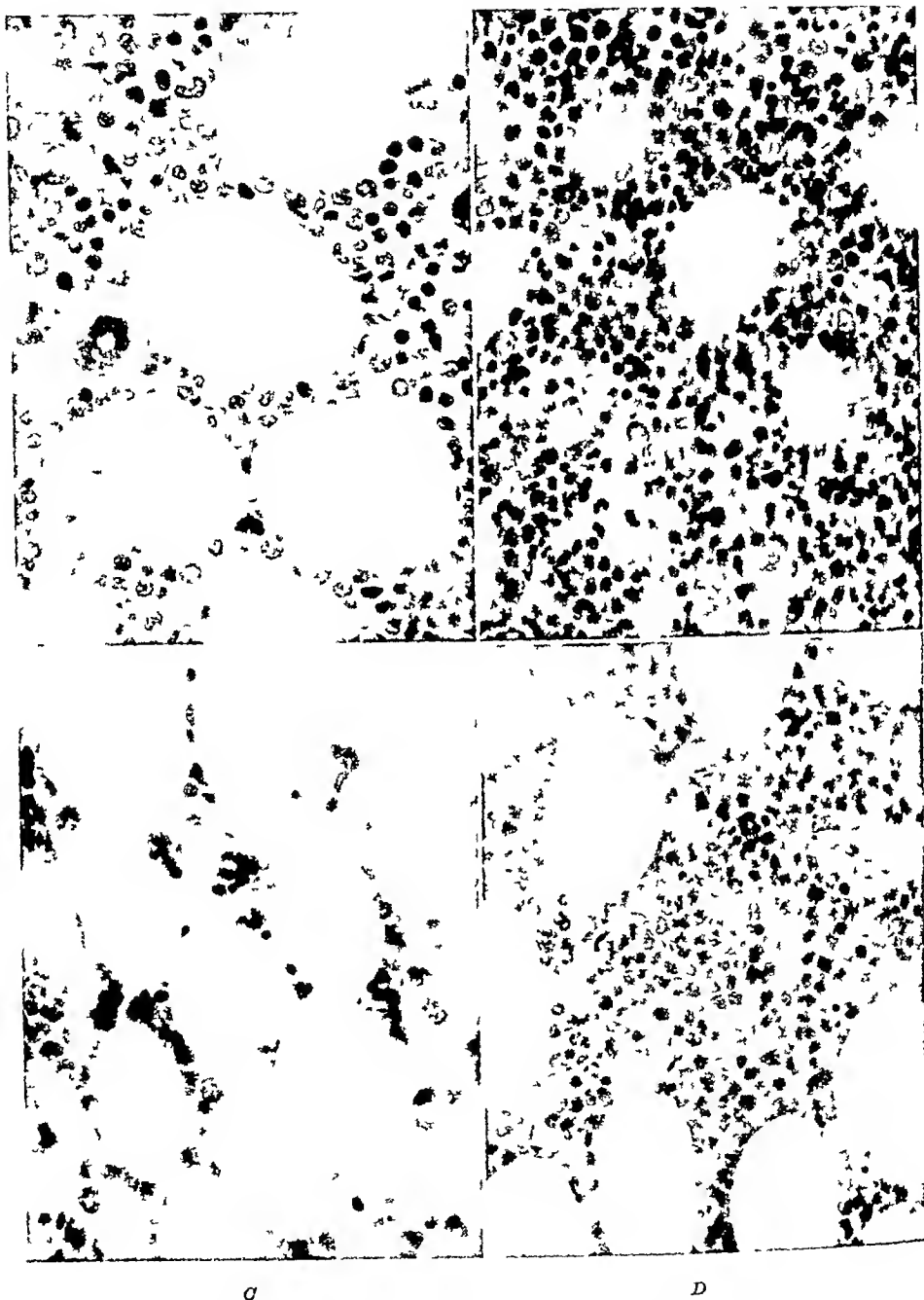


Fig 9—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine ( $\times 420$ )

A, Normal

B, Eight days after last injection of phenylhydrazine showing persistence of hyperplasia which is largely erythropoietic

C, Six days after intravenous injection of nitrogen mustard (3 mg per kilogram) Regeneration is progressing at this stage.

D, Six days after intravenous injection of nitrogen mustard (3 mg per kilogram) and eight days after phenylhydrazine withdrawal. Hyperplasia involves granulopoietic and erythropoietic series

*Period of Regeneration (Five Days Through Fourteen Days)*—The marrow of animals which received HN only (Group 2) contained scattered islands of hemocytoblasts and myelocytes and erythroblasts in various stages of maturation on the fifth day after the HN injection and increasingly greater numbers on the sixth and seventh day intervals. On the eighth and ninth day intervals granulocytes and megakaryocytes became more conspicuous. This more or less orderly regeneration continued by the fourteenth day the marrow was essentially similar to that of the untreated controls of Group 1. This period of active regeneration is illustrated in Figs 9 and 10. In the marrow of animals which received phenylhydrazine prior to the HN<sub>2</sub> injection (Group 4), erythroblastic hyperplasia continued to be active far in excess of Group 2 (HN only) or Group 1 (normal) and only gradually returned to a normal state. Whereas evidence of granulo and megakaryocytopoietic regeneration was conspicuous on the eighth day in the animals that received HN only (Group 2) active regeneration of granulo and megakaryocytopoietic cells was evident beginning on the fourth day and increased through the ninth day in the animals which received phenylhydrazine and HN<sub>2</sub> (Group 4). The intense erythroblastic proliferation seen at previous intervals continued with only slight diminution during this period.

*Comparison of X ray and HN on Phenylhydrazine Induced Anemia and Hyperplastic Marrow*—In a previous series of papers<sup>9</sup> the effects of 800 r whole body x irradiation on the peripheral blood and bone marrow of normal rabbits and rabbits with a phenylhydrazine induced anemia have been described in detail. The conditions of these experiments were identical with those of the experiments described in this paper except that a physical agent (800 r x ray) was given instead of a chemical agent (HN). Although the main findings in the x ray experiments are essentially the same as in the HN experiment, certain gross differences make a brief comparison worthwhile. As was demonstrated in Figs 1 and 2 nitrogen mustard alone produced an anemia which was on the borderline of significance some two weeks or more after the HN administration. Animals which had a phenylhydrazine induced regenerative anemia developed further anemia within the first five days after HN<sub>2</sub> administration. A dose of 800 r whole body x irradiation produced an anemia in rabbits which reached a maximum on about the fourteenth day (Fig. 11). On the other hand, the administration of 800 r to animals with a phenylhydrazine induced regenerative anemia produced neither an early nor a latent anemia and recovery proceeded rapidly but not as rapidly as occurred in the phenylhydrazine and HN animals (Fig. 12). This difference in the early effect of phenylhydrazine followed by HN on the one hand and phenylhydrazine followed by x irradiation on the other was not apparent in the bone marrow since in neither was erythrocytopoiesis seriously interrupted.

Nitrogen mustard alone and x ray alone produced a comparable degree of cellular depletion in the marrow of normal rabbits but bone marrow recovery was more rapid in the HN series than in the x ray series (Compare Fig 9, C with Fig. 13 1). Similarly the degree of cellular depletion in the marrow

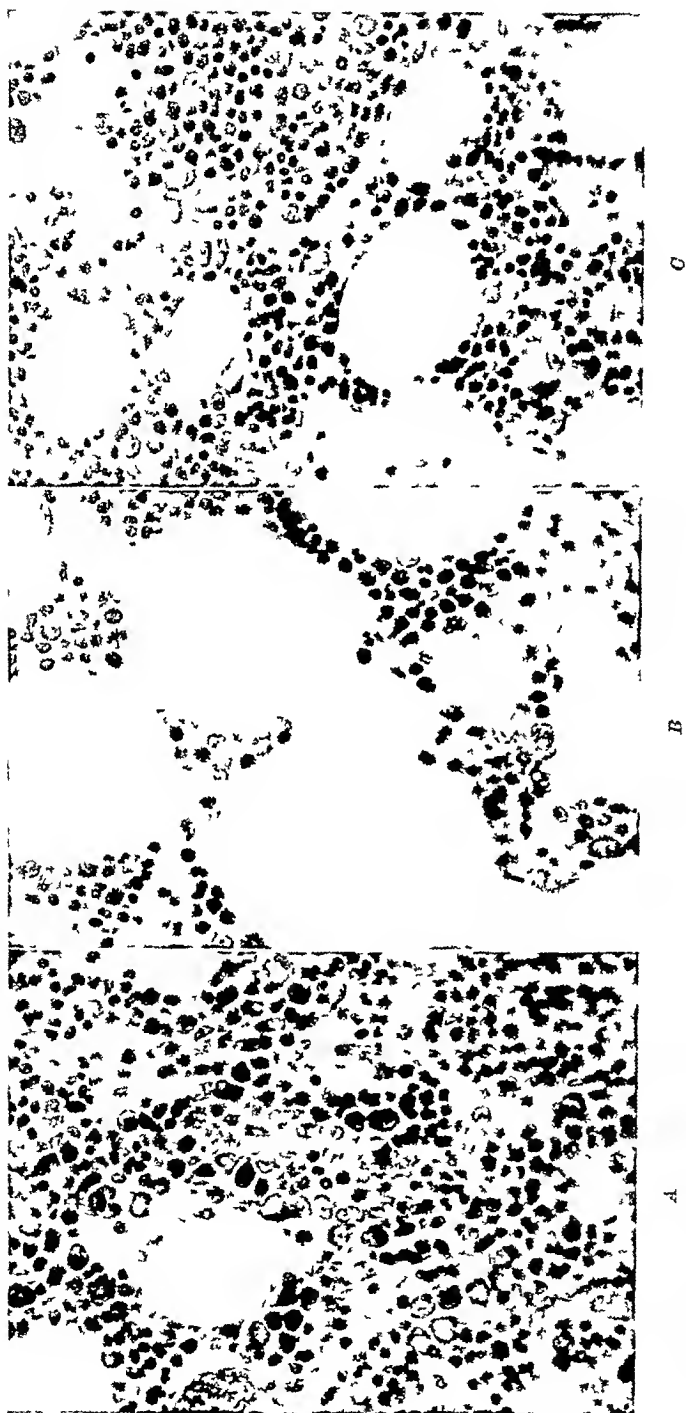


Fig 10.—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine ( $\times 120$ )

A, Eleven days after last injection of phenylhydrazine

B, Nine days after intravenous injection of nitrogen mustard (3 mg per kilogram) Marrow essentially normal

C, Nine days after intravenous injection of nitrogen mustard (3 mg per kilogram) and eleven days after phenylhydrazine withdrawal Marrow now essentially normal except that general hyperplasia persists

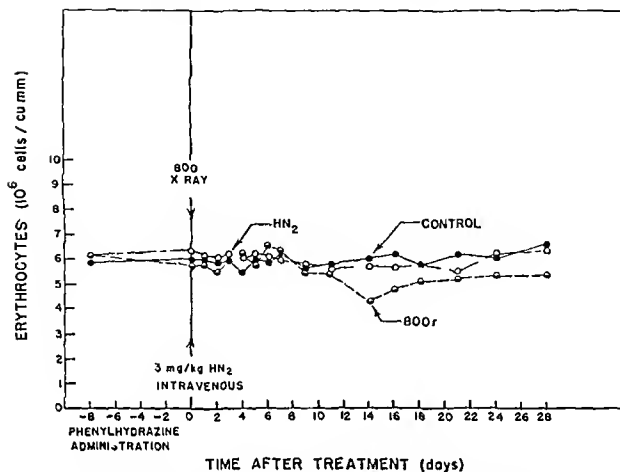


Fig 11—Comparative effect of 800 r whole body x irradiation and 3 mg per kilogram nitrogen mustard on the circulating erythrocytes of normal rabbits

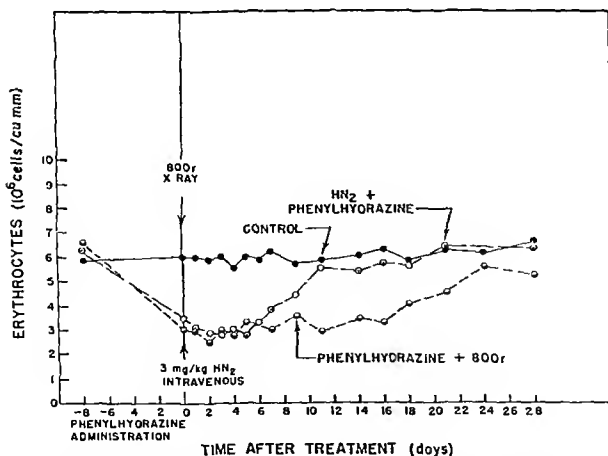
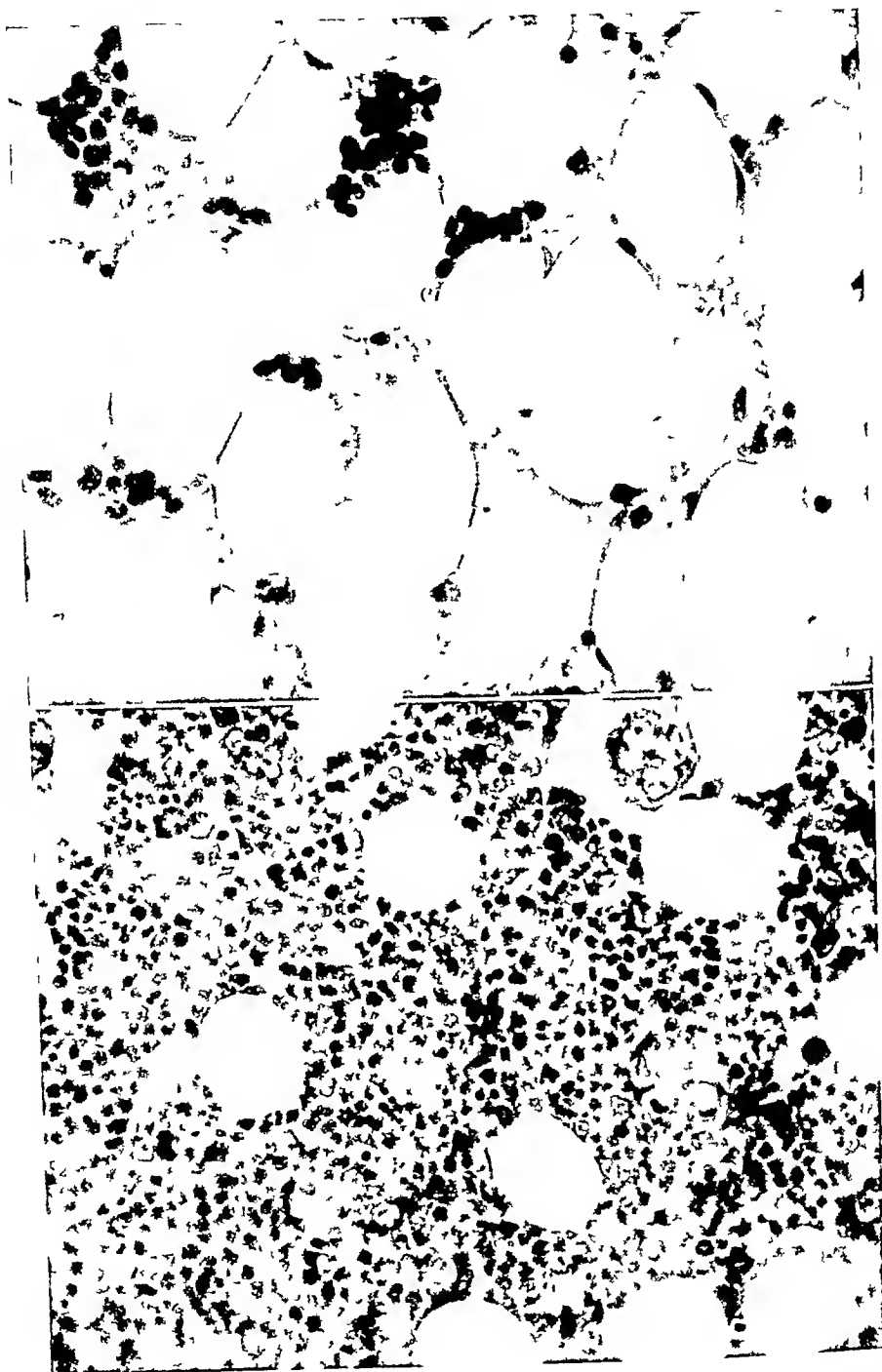


Fig 12—Comparative effect of 800 r whole body x irradiation and nitrogen mustard (3 mg per kilogram) on the erythrocyte values of rabbits which had previously been made anemic by phenylhydrazine administration

A



B

Fig 13—The effect of 800 r whole body x-irradiation on the femoral marrow of a normal rabbit and on rabbits which had previously received phenylhydrazine ( $\times 420$ )  
 A, Six days after 800 r whole body x-irradiation. Except for small islands of erythrocytopoiesis the marrow is still atrophic.  
 B, Six days after 800 r whole body x-irradiation and eight days after last injection of phenylhydrazine. This shows the marked hyperplasia which is found scattered about in the marrow.



of animals which received both phenylhydrazine and HN was comparable with the cellular depletion in the bone marrow of animals which received both phenylhydrazine and  $\gamma$  ray in the early stages (one day after HN or  $\gamma$  ray respectively). However, the marrow of the phenylhydrazine HN<sub>2</sub> animals underwent a more rapid regeneration than that of the phenylhydrazine  $\gamma$  ray animals.

#### DISCUSSION

It is interesting that the production of a regenerative anemia with phenylhydrazine or bleeding protects against the usual latent radiation induced anemia in rabbits by virtue of the survival of sufficient hematopoietic tissue to insure an almost immediate hyperplastic reaction.<sup>6,7</sup> Stimulation of erythropoiesis likewise protects the marrow from the destruction regularly produced in rabbits by the chemical agent methyl bis ( $\beta$  chloroethyl) amine hydrochloride. The degree of destruction of the marrow which regularly follows the administration of a single intravenous dose of 3 mg of HN<sub>2</sub> or 800 r whole body  $\gamma$  irradiation is comparable from the histologic point of view through the fourth day after exposure to either of these agents. A significant anemia follows the latter (800 r) which reaches its maximum on the fourteenth day after exposure whereas an anemia of only borderline significance is apparent after the former (HN). As is evident in the data presented, the histologic recovery of the marrow from intoxication with HN<sub>2</sub> only is so rapid that the brief but complete cessation of erythropoiesis is essentially masked.

The further fall of the erythrocyte values and hemoglobin, after HN<sub>2</sub> injection in animals already made anemic with phenylhydrazine (Group 4), is very likely due to continued hemolysis as a result of the combination of phenylhydrazine and HN. No evidence of significant hemolysis was apparent during this initial period in animals which received HN<sub>2</sub> only (Group 2), nor did further significant anemia develop in animals which had received only phenylhydrazine (Group 3). In previous experiments in which phenylhydrazine administration was followed by 800 r whole body  $\gamma$  irradiation, no further anemia developed immediately following  $\gamma$  ray (one to five days). It is therefore suggested that phenylhydrazine plus HN exerts a synergistic hemolytic effect on circulating erythrocytes. After reaching a minimum of less than  $2.8 \times 10^6$  per cubic millimeter, the erythrocytes of the animals which had phenylhydrazine and HN<sub>2</sub> rose so rapidly that a normal value was evident as rapidly as in the animals which received phenylhydrazine only (Group 3) and before the value of the group which received HN only had fallen to its lowest point. The histologic findings indicate that a sufficient number of erythroblasts and hemocytoblasts survive the insult of the HN<sub>2</sub> in the latter group to immediately carry on normal erythropoietic activity and subsequently proceed to an intense hyperplasia and rapid recovery. In the group which received HN<sub>2</sub> only (Group 2) on the other hand erythropoietic tissue was essentially destroyed and compensatory hyperplasia and recovery were comparatively slow. In previous reports<sup>2,3</sup> it was pointed out that whereas mitotic activity stopped in the bone marrow of normal rabbits during the first twenty four hours after 800 r whole

body  $\alpha$ -radiation, mitotic activity in the marrow of animals with an erythroblastic hyperplasia continued at a normal or greater than normal rate after this dose of  $\alpha$ -radiation. The findings in the present study with  $\text{HN}_2$  are identical in this respect with the  $\alpha$ -ray studies. The free cells of the hematopoietic marrow which survive the insult of  $\text{HN}_2$  are largely hemocytoblasts and basophilic erythroblasts. This fact is good evidence for support of the concept that the less mature cells are more resistant than more mature cells of the erythroblast series. If the more mature forms were less sensitive or of equal sensitivity to their precursors, one would expect to find a proportionate number of various maturation phases surviving at the early intervals after  $\text{HN}_2$  particularly. In other words, one would expect surviving erythroblasts in each maturation phase to be in a proportion or ratio to one another more nearly like that of the hyperplastic marrow at the time of  $\text{HN}_2$  administration if the sensitivity were equal. If the sensitivity of various maturation phases differs, then a predominance of the more resistant forms would be found in the early intervals after exposure to the noxious agent. Since the surviving free cells were predominantly basophilic erythroblasts and hemocytoblasts, one must conclude that under the conditions of this experiment these immature cells are less sensitive to  $\text{HN}_2$  than the more mature forms in the marrow. It is further apparent that reticular cells not only retain their function of transformation to the free precursors of the marrow but also that the stimulation provided by the phenylhydrazine administration has probably increased this transformation to an extent which is significant in the development of the rapid hyperplasia and recovery.

Of interest is the fact that after  $\text{HN}_2$  only (Group 2), granulocytopoietic, megakaryocytopoietic, and erythrocytopoietic tissue in the marrow is more completely destroyed than lymphatic tissue in the spleen, lymph node, and thymus. Recovery of the bone marrow and return of the circulating granulocytes, erythrocytes, and platelets to normal values, however, precede the complete histologic recovery of lymphatic tissue and the return of lymphocyte values of the peripheral blood to normal.

It is of even greater interest that recovery of the bone marrow from the insult of  $\text{HN}_2$  (3 mg per kilogram) is more rapid than the recovery from  $\alpha$ -ray (800 r) even though the degree of destruction and atrophy is entirely comparable during the early phases (one to four days). If one assumes that this difference in recovery rate is on the basis of the fact that  $\text{HN}_2$  has a more selective action than total body  $\alpha$ -radiation, then one is assuming that irradiation can produce an indirect suppression of marrow hematopoiesis or that irradiation produces greater effects on reticular cells which retard heteroplastic blood formation than does  $\text{HN}_2$ . The latter possibility seems more likely.

Significant regeneration of granulocytopoiesis and megakaryocytopoiesis in the marrow of animals which received phenylhydrazine and  $\text{HN}_2$  (Group 4) occurred at a distinctly earlier stage than in the animals which received  $\text{HN}_2$  only (Group 2). This regeneration occurs homoplastically from a few surviving myelocytes and heteroplastically from reticular cells and hemocytoblasts. This

histologic evidence of an earlier regeneration of granulocytes in the animals which received both phenylhydrazine and HN (Group 4) correlates well with the studies on the peripheral blood as illustrated in Fig 5

The degree of destruction and time required for recovery of lymphatic tissue after the administration of HN only (Group 2) appear to be entirely comparable with the destruction and recovery of lymphatic tissue after phenylhydrazine and HN (Group 4)

These observations suggest that the production of a hyperplastic marrow prior to the use of a irradiation or HN might be useful as a means of protecting the normal hematopoietic tissue when these agents are used in therapy of such neoplastic disorders as the lymphomas. The fact that the persistent hyperplasia after HN<sub>2</sub> or x ray is dependent in part on heteroplastic formation of the free marrow precursors from reticular cells suggests that venesection almost immediately before HN or x ray administration might effect a more rapid regeneration of the marrow from the destructive action of these agents. Phenylhydrazine administration would not seem particularly desirable under these conditions but perhaps adequate and controlled stimulation could be achieved by exposure to a low atmosphere of oxygen or venesection

#### SUMMARY

The effect of intravenous administration of a nitrogen mustard (methyl bis ( $\beta$  chloroethyl) amine hydrochloride) in a dose of 3 mg per kilogram was studied in normal rabbits and in rabbits with an anemia and erythroblastic hyperplasia produced by phenylhydrazine hydrochloride. Controls consisted of normal untreated rabbits and rabbits given phenylhydrazine alone. Histopathologic studies were made of animals from each group which were sacrificed at frequent intervals. Hematologic observations likewise were made at frequent intervals.

A single intravenous injection of HN produced only a modest anemia of questionable significance in normal rabbits which was maximal at approximately three weeks. Leucopenia and a marked reduction in the number of circulating reticulocytes also were produced. A rapid destruction of hematopoietic tissue in the spleen, thymus, lymph nodes, and bone marrow occurred within twenty-four hours. The virtual absence of mitoses during the first twenty-four hours after the injection of HN was striking; in stages up to the fifth day mitoses were seen only rarely. During the second, third, and fourth days after HN the bone marrow was practically devoid of cells. Only a few scattered hemierythroblasts, erythroblasts, plasma cells, and giant myelocytes were seen. Erythropoietic, granulopoietic, and megakaryocytopoietic recovery in the group occurred more or less simultaneously. Regeneration evidence of which was first apparent on the fifth day progressed to an essentially normal marrow by the tenth day. Lymphatic tissue though severely damaged by HN<sub>2</sub> was less atrophic than the bone marrow but regeneration was slower and reconstruction was not apparent until after the fourteenth day. The appearance of leucopenia and reticulocytopenia correlated closely with the hematopoietic destruction, atrophy, and eventual reconstitution. The anemia which developed was modest, probably because of the rapid reconstitution of erythropoiesis in the marrow.

Phenylhydrazine alone in a dose totaling 40 mg produced an approximate 50 per cent reduction in erythrocyte and hemoglobin values with a simultaneous reticulocytosis and erythroblastic hyperplasia in the bone marrow. These hematologic values of the peripheral blood returned to normal by the twelfth day after phenylhydrazine withdrawal, but increased erythrocytopoiesis in the marrow persisted for about two weeks.

Animals to which 3 mg per kilogram of  $\text{HN}_2$  were given after an anemia and an erythroblastic hyperplasia of the bone marrow had been produced by phenylhydrazine developed further anemia within the first five days after the  $\text{HN}_2$  administration, but recovery was complete by the twelfth day. *In fact, the hemoglobin and erythrocyte values of this group returned to normal as quickly as in animals which were given phenylhydrazine only.* The reticulocyte value of the animals which received phenylhydrazine prior to the  $\text{HN}_2$  fell from a value of circa 25 per cent to 2 per cent by the third day after  $\text{HN}_2$ , but rose rapidly to 15 per cent by the sixth day and slowly fell to normal by about the fourteenth day. The marrow of these animals was extremely hyperplastic at the time at which the  $\text{HN}_2$  was given. The usual evidence of destruction followed the  $\text{HN}_2$  administration, but large numbers of cells in the bone marrow survived as compared with animals which were given  $\text{HN}_2$  only. *The surviving cells were predominantly basophilic erythroblasts and hemocytoblasts and were present in an appreciably larger number than normally present in controls.* Heteroplastic regeneration from reticular cells was conspicuous throughout the first six days after  $\text{HN}_2$  administration. Mitoses were present in large numbers even in the early stages after the  $\text{HN}_2$  administration and increased rapidly to a maximum by about the fourth day. The more mature forms of the erythroblast series were progressively increased in the second, third, fourth, fifth, and sixth day stages after  $\text{HN}_2$ . A significant recovery of granulopoiesis and megakaryocytopoiesis in the marrow was apparent beginning on the fourth and fifth day after  $\text{HN}_2$  in these animals. Hyperplasia in the marrow of these animals continued for about two weeks.

The fact that further anemia followed immediately after  $\text{HN}_2$  administration in the group of animals which received phenylhydrazine and  $\text{HN}_2$  cannot be interpreted as related to bone marrow destruction since a normal or greater than normal amount of erythrocytopoiesis was present during this period as evidenced by the histologic studies and the increased number of reticulocytes in the peripheral blood. It seems likely that this was a hemolytic effect due to a synergistic action of phenylhydrazine and  $\text{HN}_2$  under the conditions of this experiment.

The initial effects of 800 r whole body x-radiation on the hematopoietic system of normal animals and animals with a regenerative anemia and hyperplastic bone marrow are largely comparable with the initial effects produced by  $\text{HN}_2$  insofar as the destruction of the bone marrow is concerned. The latter agent ( $\text{HN}_2$ ), however, apparently intensifies the hemolytic action of phenylhydrazine on circulating erythrocytes, whereas x-ray does not. Regeneration of the bone marrow after  $\text{HN}_2$  alone or phenylhydrazine plus  $\text{HN}_2$  is more rapid than after x-ray alone or after phenylhydrazine plus x-ray.

It is suggested that stimulation of the hematopoietic system of the human being with disorders such as lymphomata might be feasible as a means of partial protection of or hastening the recovery of normal hematopoiesis from such destructive therapeutic agents as x ray or HN

#### CONCLUSIONS

1 In normal rabbits the intravenous injection of nitrogen mustard (methyl bis ( $\beta$  chloroethyl) amine hydrochloride) in a dose of 3 mg per kilogram body weight produces a modest anemia and a severe leucopenia and reticulocytopenia

2 Nitrogen mustard in a dose of 3 mg per kilogram when given to animals in which an anemia, reticulocytosis and hyperplastic marrow are already present by virtue of prior phenylhydrazine hydrochloride administration produces

(a) Further anemia within the first five days after the HN administration which is presumed to be due to an apparent synergistic hemolytic action of phenylhydrazine and HN on the circulating erythrocytes. No latent anemia is produced under these circumstances such as is produced by  $\text{HN}_2$  alone. Recovery from the anemia induced by phenylhydrazine and HN is as rapid as from the anemia produced by phenylhydrazine alone and faster than the recovery from the modest anemia produced by HN alone

(b) A leucopenia comparable with that produced by HN only. Recovery from the leucopenia induced by phenylhydrazine and HN is more rapid than the recovery after HN only

(c) A reduction of the reticulocyte value from circa 25 per cent to the normal value by the third day followed by immediate recurrence of the reticulocytosis.  $\text{HN}_2$  alone, on the other hand, produces a marked reduction below normal values with a gradual return to a normal value

3 In the normal rabbit  $\text{HN}_2$  alone produces a marked depletion of lymphatic tissue recovery from which requires more than two weeks. Bone marrow becomes extremely atrophic but regeneration begins by the fifth day. Mitotic activity which is virtually absent in these tissues for the first twenty four to forty eight hours after HN administration is only gradually resumed

In rabbits with an anemia, reticulocytosis and hyperplastic marrow induced by phenylhydrazine hydrochloride the intravenous injection of 3 mg of nitrogen mustard per kilogram of body weight produces the usual evidence of destructive effects in the hematopoietic tissue but large numbers of hemocytoblasts and erythroblasts in the bone marrow especially survive which are immediately capable of proliferation. Mitotic activity continues at a normal or greater than normal rate under these conditions

4 The production of a hyperplastic (erythroblastic) marrow protects the marrow from the destructive effects of HN by virtue of increasing the population of hemocytoblasts and basophilic erythroblasts which are less sensitive to this chemical agent than the more mature derivatives of these cells. Heteroplastic regeneration from reticular cells likewise probably plays a significant role in the rapidity of recovery

5 More immature forms of the erythroblast series and hemocytoblasts are less sensitive to either a physical agent (X-ray) or a chemical agent (HN<sub>2</sub>) than are the more mature forms of the same maturation series

6 Prior stimulation of hematopoiesis in the human being as a means of partially protecting the hematopoietic tissue from such therapeutic agents as nitrogen mustard or irradiation may be feasible

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## DIETARY AND HORMONAL INFLUENCES IN EXPERIMENTAL UREMIA

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THE mechanism of uremia has not been defined in precise chemical terms. Still, on general grounds treatment is directed toward control of azotemia and hyperkalemia. The most obvious means to these ends are dietary and consist in restriction of exogenous protein and potassium and in the administration of high caloric diets consisting of carbohydrate and fat. Such diets slow the rate of endogenous protein catabolism and also inhibit the release of potassium from the cell stores. The advantages of such a regime supplemented with blood transfusion and by antibiotics has been demonstrated recently by Boist.<sup>1</sup>

In addition theoretical and experimental considerations suggest that advantage might be taken of the actions of certain hormones. Thus desoxy corticosterone acetate (DCA) prolongs life when given to animals prior to bilateral nephrectomy,<sup>2,3</sup> and an anturemic property probably dependent on increased protein anabolism<sup>4</sup> has been ascribed to testosterone.<sup>5</sup> Lastly, insulin since it decreases potassemia and tends to slow the conversion of protein to glucose, might be of some value.<sup>6</sup>

While the general considerations underlying the treatment of uremia by high caloric nonprotein diets are entirely clear and valid a survey of the field indicated a dearth of critically controlled experimental comparisons in significantly large groups of animals. The present study was therefore designed for the evaluation of the comparative effects of isocaloric diets of protein fat and carbohydrate on the survival time of rats subjected to bilateral nephrectomy and of the effect of hormones under controlled conditions of diet and fluid intake.

The first series of observations were made in normal animals and the second in animals in which infection was simulated by formation of turpentine abscesses. The distinction is important since infection with its acceleration of protein catabolism and discharge of protein metal oxides and potassium into the blood is a frequent complication in uremic patients.

### SERIES I NORMAL ANIMALS, BILATERALLY NEPHRECTOMIZED EFFECTS OF DIET AND HORMONES

(a) *Method*—The animals used were male albino rats weighing 200 to 350 grams. The experimental groups were selected for equal weights in each because age is a determinant of survival after nephrectomy.<sup>7,10</sup> Nephrectomy was done in two stages at intervals of

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seven days Treatment was begun immediately after removal of the second kidney Effects of each of the diets and of concurrent hormonal treatments were tested separately and, in each of the comparative series, a reference group given either protein or carbohydrate was used The carbohydrate and fat diets were prepared in the manner of Remncke, Ball, and Samuels<sup>11</sup> but without salt mixture and vitamins, the protein diet used was isocaloric and consisted of egg albumin, casein, and a protein digest The diets were given twice daily by gavage in a daily volume of 3 cc per 100 sq cm of body surface In each of the diets, this volume contained 10 calories Body surface was calculated from the formula  $S = KW^{.66}$  where  $S$  is body surface in square centimeters,  $K$  a constant of 9.1, and  $W$  the weight in grams The potassium contents of the diets were measured by the method of Shohl and Bennett<sup>12</sup> and found to be 1.1, 0.29, and 0.84 mg per cubic centimeter respectively for protein, fat, and carbohydrate rations

Steroid hormones were given subcutaneously in daily doses of 10 mg of 0.4 cc hydro alcoholic suspension Blood samples of 0.2 cc were taken from the tail at thirty and forty eight hours after nephrectomy for determination of urea nitrogen by the method of Archibald<sup>13</sup>

(b) *Results*—The effects of treatment on survival time and on the level of blood urea nitrogen are summarized in Table I

Inspection of the data shows first that survival decreases as azotemia increases Second, comparison of Groups 1 (fasting) and 2 (protein-fed) shows that fasting, contrary to some impressions, is as deleterious as protein feeding Third, comparisons of Groups 3 and 7, 8 and 12, and 13 and 17 indicate that survival is longer and azotemia less in carbohydrate- and fat-fed than in protein-fed animals Last, survival is somewhat shorter and azotemia greater in animals given fat instead of carbohydrate, a difference which can be attributed to the diarrhea caused by a pure fat diet

Treatment with hormones, singly or in combination, had no significant effect on survival and azotemia in animals fed either protein or fat However, in animals fed only carbohydrate, blood urea nitrogen levels at thirty hours after nephrectomy were somewhat lower during treatment with DCA (Group 9) and testosterone (Group 10) than in the controls The difference was even greater in animals given combined treatment of the two steroids (Group 11) Mean survival time was somewhat prolonged by hormonal treatment To confirm this observation, the experiments were repeated on four groups (8a, 9a, 10a, and 11a) of ten animals each, which were treated exactly as were animals in Groups 8, 9, 10, and 11 In these groups blood urea nitrogen concentrations at thirty hours after nephrectomy were as follows: group 8a, 129 (range, 94 to 150) mg per 100 cc, group 9a, 92 (71 to 100), group 10a, 112 (78 to 150), and group 11a, 70 (57 to 87) Thus, these observations establish the facts that testosterone and, curiously, DCA both tend to suppress azotemia in carbohydrate-fed, nephrectomized animals

#### SERIES II ANIMALS WITH SIMULATED INFECTION (TURPENTINE ABSCESS)

The purpose of this series of experiments was to test the effects of carbohydrate feeding as such and of the same diet when combined with steroid hormones and insulin under conditions of catabolic stress



TABLE I. MEAN SURVIVAL TIME AND BLOOD UREA NITROGEN LEVEL IN BILATERALLY NEPHRECTOMIZED RATS

GROUP	DIET	TREATMENT	NUMBER OF RATS	SURVIVAL TIME (HR)	BLOOD UREA NITROGEN (MG. %)	
					AT 30 HF	AT 48 HF
1	--	--	16	48 (44-64)	297 (200-75)	388 (337-430)
2	Protein	--	10	60 (44-82)	154 (140-192)	300 (245-325)
3	Protein	DCA	20	40 (36-68)	50 (45-50)	540 (51-575)
4	Protein	Protein	10	44 (34-57)	203 (216-29)	543 (460-605)
5	Protein	Testosterone	10	44 (31-57)	240 (194-30)	461 (400-512)
6	Protein	DCA and testosterone	10	52 (38-60)	281 (253-312)	510 (460-562)
7	Carbohydrate	--	10	86 (60-108)	167 (140-185)	237 (205-262)
8	Carbohydrate	--	20	93 (59-128)	112 (74-152)	134 (80-157)
9	Carbohydrate	DCA	9	127 (83-179)	89 (69-106)	131 (105-146)
10	Carbohydrate	Testosterone	9	118 (60-156)	100 (73-152)	130 (95-173)
11	Carbohydrate	DCA and testosterone	9	116 (70-156)	00 (46-88)	90 (67-115)
12	Protein	--	9	53 (44-68)	215 (162-282)	--
13	Fat	--	20	77 (52-98)	175 (150-205)	208 (218-322)
14	Fat	DCA	18	70 (50-100)	167 (112-28)	210 (172-282)
15	Fat	Testosterone	18	74 (57-103)	173 (158-193)	298 (282-343)
16	Fat	DCA and testosterone	18	75 (39-100)	166 (147-191)	247 (238-255)
17	Protein	--	10	40 (41-53)	-39 (167-285)	455 (438-468)

(a) *Method*—Thirty rats were divided into five groups. Following a two stage nephrectomy, all the animals were fed a high carbohydrate diet by stomach tube. Supplementary treatment was started as indicated in Table II and turpentine was injected once subcutaneously into the prepectoral region in a dose of 0.5 cc in four of the groups. Prothamine zinc insulin was given in a dose of 1 unit daily in two groups and the steroid hormones were given in doses of 5 mg twice a day in another two groups. Blood urea nitrogen was determined thirty and forty eight hours after nephrectomy. At forty eight hours the animals were anesthetized and blood was collected for potassium determination.<sup>18</sup> Data are collected in Table II.

TABLE II INFLUENCE OF INSULIN AND STEROID HORMONES ON THE MORTALITY AND BLOOD UREA NITROGEN OF NEPHRECTOMIZED RATS INJECTED WITH TURPENTINE AND FED CARBOHYDRATE

TREATMENT	BLOOD UREA NITROGEN (MG PER 100 CC)		SERUM POTASSIUM (MG PER 100 CC)	NUMBER OF DEATHS AT 48 HR
	AT 30 HR	AT 48 HR		
--	111 (74-142)	156 (125-186)	35	0
Turpentine	156 (127-172)	242 (202-275)	45	1
Turpentine and insulin	182 (157-204)	241 (210-280)	42	1
Turpentine and insulin and DCA and testosterone	179 (167-215)	252 (217-277)	43	3
Turpentine and DCA and testosterone	174 (147-202)	238 (210-272)	46	1

(b) *Results*—The increased azotemia and mortality at forty-eight hours consequent on turpentine abscesses were not influenced by hormonal treatment. Furthermore, animals which were not given turpentine (group 1) remained in good condition (temperature, muscular tone, reactivity), while those with turpentine abscesses were apathetic, asthenic, cold, and had buccal hemorrhages. Morbidity and mortality corresponded roughly with the serum potassium contents. Therefore, under the present experimental conditions, neither steroid hormones nor insulin proved to be of any advantage over feeding a high carbohydrate high caloric diet alone, and the diet did not prevent the deleterious effects of simulated infection.

#### DISCUSSION

In earlier studies, Lyon<sup>1</sup> and Lyon, Shafton, and Ivy<sup>16</sup> showed that the mean survival time of nephrectomized dogs fed on bread, milk, and lactose was 137.6 hours as compared with 105.6 hours for dogs fed on a meat diet. In contrast Winternitz, Mylon, Waters, and Katzenstein<sup>17</sup> reported no beneficial effects of a carbohydrate feeding to nephrectomized dogs, taking as criteria of benefit blood urea and survival time. Their animals, however, were treated with fluids and alkalis, in order to maintain normal blood volume and alkaline reserve. There is no proof that such procedures are of real benefit, while it is well recognized that anuric animals do not withstand excess fluid or electrolytes. Such treatment might therefore obscure any effect of diet. The harmful effects of water have been demonstrated by Bergman and Dimy<sup>9</sup> and by Burne, Eversole, and Gaunt.<sup>10</sup> Bergman and Dimy<sup>9</sup> also showed that preoperative fasting does not influence significantly the survival time, and that meat is definitely toxic while glucose improves the condition of the rats.

They obtained a definite prolongation of survival time by daily administration of as little as 5 cc of a 20 per cent glucose solution. Since this represents only 45 calories in contrast to the average daily intake of about 50 calories given in our experiments it seems carbohydrate is beneficial even though it does not meet the caloric requirements. Mylon and Goldstein<sup>18</sup> found also that preoperative feeding of a carbohydrate diet to dogs subjected to nephrectomy or renal artery ligation prolongs the survival time and delays the blood nonprotein nitrogen rise which occurs with preoperative meat feeding. The toxic effects of protein in experimental uremia produced by ligation of the vena cava above the renal veins have also been demonstrated by Addis and Lew.<sup>19</sup> More recently Addis<sup>20</sup> showed that protein diet has a deleterious influence in animals subjected to sudden loss of three fourths of the renal tissue.

These results and our own confirm the well established principles that high caloric carbohydrate and fat diets have an inhibitory effect on protein catabolism which in turn delays or diminishes uremia.

DCA given preoperatively has been shown to prolong the survival time of nephrectomized rats and mice.<sup>2, 3, 4</sup> However Winkler, Smith and Hoff<sup>21</sup> were unable to demonstrate any beneficial effect from DCA or adrenocortical extract given postoperatively to dogs made urmic by metarterial ligation. They suggested that the probable protective mechanism of DCA pretreatment lies in its depletion of the body stores of potassium. That this may be so is shown by the increased survival of nephrectomized animals preoperatively depleted of potassium by diet alone.<sup>2, 3</sup> Rodbard<sup>5</sup> has confirmed the fact that the life of nephrectomized dogs can be prolonged by preoperative treatment with DCA. He also observed some beneficial effect from DCA given postoperatively which was unrelated to the level of serum potassium. Our data also indicate a slight effect on survival and a more definite decrease in azotemia from post operative treatment with DCA and testosterone in rats fed only on carbohydrate and thus confirm Selye and Nielsen.<sup>4</sup> As concerns DCA, Selye<sup>6</sup> has also observed a beneficial effect of testosterone given pre and postoperatively to nephrectomized mice. Since the effect cannot be ascribed to renal potassium depletion,<sup>5</sup> it must depend on the protein anabolic property of the steroid.

Our data emphasize the importance of diet in experiments on urmic animals. They also suggest that some of the contradictory results of post operative treatment with hormones as to survival time and blood nonprotein nitrogen can be attributed to differences in the diets on which the different groups of animals were maintained in the several laboratories. While the dietary data form a basis for chemical treatment the hormonal effects obtained during treatment with massive doses can hardly serve as recommendations for a clinical trial.

Finally although high carbohydrate and fat diets can prolong survival and diminish the rate of increase of azotemia in nephrectomized animals they are ineffective in countering the protein catabolic impulse caused by tumor time abseess. This is a confirmation of Boirst's observation<sup>1</sup> that the effective

ness of dietary treatment depends on the control of infection. These and other aspects of the treatment of uremia have been reviewed editorially<sup>5</sup> and the recommendation has been made that Boist's protein-free regime has more theoretical merit than the low protein ration of Black and Stanbury.<sup>27</sup>

#### SUMMARY

Effects of fasting and various high isocaloric diets consisting of carbohydrate, fat, or protein were tested in bilaterally nephrectomized rats.

Fasting is not more advantageous than protein feeding. Carbohydrate and fat are definitely more beneficial in terms of survival and azotemia than protein diet and, of the two, carbohydrate feeding is better tolerated.

Desoxycorticosterone acetate and testosterone alone or in combination were somewhat effective only in rats fed a carbohydrate diet, the most significant change was a decrease of azotemia following combined treatment with both steroids.

High carbohydrate diet alone or in combination with DCA, testosterone, or insulin did not influence the course of uremia in nephrectomized rats subjected to turpentine abscess.

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## EFFECT OF AN ACID AND ALKALINE SALT ON THE URINARY EXCRETION OF IRON

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MUCH work has been done on the absorption, utilization, and excretion of iron, and although new information is accumulating there is still confusion in respect to many of the metabolic processes concerned. The amount of iron ordinarily excreted in the urine is small,<sup>1-3</sup> although a study on 100 men and 100 women<sup>4</sup> showed a greater excretion than had many of the previous investigations and also revealed a higher urinary iron excretion in women than in men, 0.489 mg. per day as compared with 0.395 milligram. The urinary iron excretion was found to be fairly constant in the same individual from day to day. There is a marked and prompt increase in the urinary iron excretion following intravenous administration of iron<sup>5,6</sup> although no appreciable increase occurs in the iron content of the feces under these circumstances. Little, Power, and Wakefield<sup>5</sup> thought that this was due to the marked and rapid increase in the serum iron concentration, and that it probably did not occur with normal iron absorption from the intestine. Our previous investigation<sup>4</sup> did not show an increase in the urinary iron excretion when large amounts of iron were given by mouth or with intramuscular iron injections given to the point of tolerance. Although previous extensive studies have failed to clarify all phases of iron metabolism, it appears that iron is absorbed more readily when in a ferrous state than as a ferric salt, that an acid reaction in the stomach favors the absorption of dietary iron, and that absorption is regulated by the body need.<sup>7,8</sup>

The present study was undertaken in order to ascertain the effects of an acid and an alkaline salt on the urinary excretion of iron. It has been demonstrated that an acid media in the stomach favors absorption of dietary iron, but further effects of alkalization, such as is commonly employed in the treatment of peptic ulcer, on iron metabolism have not been studied adequately.

### PROCEDURE

This study was carried out on twenty-one persons (11 men and 10 women). Ten of the subjects had peptic ulcers. The remainder were patients on the medical wards who had no disease which would interfere with intestinal absorption and who were not passing blood from the urinary or gastrointestinal tract. The subjects continued with their ordinary routine and with their hospital diets—the iron contents of which were calculated from the table of Waller.<sup>10</sup> No patients were receiving or had received iron medication so that no abnormal or sudden increase in the serum iron was induced. No medications were given aside from the alkaline or acid salts. Fluids were allowed as desired at all times.

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During the control period, twenty four hour urine specimens were collected for three days in glass containers which were especially cleaned to prevent iron contamination. Creatinine determinations were done each day to check the completeness of collection. Iron determinations were done in triplicate on aliquots of the pooled three day specimens using the method of Reis and Chakmakjian.<sup>11</sup>

Following a control period of three days the patient was given 1 Gm. of sodium bicarbonate six times a day, and twenty four hour urine specimens were collected for a three day period and handled in the same manner as in the control period. After an interval of one day during which the specimens were discarded eleven of the subjects received 1 Gm. of ammonium chloride four times a day for a three day period. Specimens were handled as before.

Hemoglobin determinations were made at the beginning of each three day period by the acid hematin method using a photoelectric colorimeter. A hematocrit was done at the same time by the Van Allen method.<sup>1</sup> All values were well within the range of normal, and the periods of observation were of too short duration to note any significant changes in hemoglobin or hematocrit values.

### RESULTS

A summary of the significant findings is presented in Table I in which the data for the male and female patients are presented separately.

TABLE I EFFECT OF AN ACID AND ALKALINE SALT ON THE URINARY EXCRETION OF IRON\*

PATIENT	AGE	BLOOD		URINE SUMMARY (AVERAGE PER DAY)								
				CONTROL			SODIUM BICARBONATE			AMMONIUM CHLORIDE		
		HEMOGLOBIN (GM)	HEMATOCRIT (%)	VOL (CC)	SP GR	Fe (MG)	VOL (CC)	SP GR	Fe (MG)	VOL (CC)	SP GR	Fe (MG)
Men												
1	43	-	-	857	1026	15	863	1024	09			
2	50	11.78	40.5	2073	1012	41	2,150	1014	21			
3	29	12.41	43.0	1997	1017	60	3,553	1012	36			
4	47	13.47	44.0	1743	1013	44	1,900	1015	24			
5	65	13.75	43.5	1220	1020	31	1,303	1019	16			
6	52	10.73	40.0	807	1021	20	1,057	1020	16	1,017	1019	38
7	31	11.41	44.5	1923	1011	38	1,607	1013	20	1,827	1014	40
8	28	12.62	41.0	650	1034	23	973	1026	19	873	1027	44
9	31	12.62	44.0	1760	1016	44	1,683	1017	34	1,287	1019	45
10	43	13.75	46.0	1827	1012	37	1,637	1012	25	2,240	1010	89
11	29	14.38	45.5	1020	1010	29	1,280	1017	22	940	1017	31
Average						35			22			49
Women												
12	24	6.38	23.0	1,631	1014	31	1,403	1016	15			
13	42	10.20	37.0	1,527	1009	23	1,580	1009	16			
14	49	10.22	39.0	2,907	1011	44	2,643	1011	26			
15	39	10.94	38.0	1,897	1013	38	1,483	1015	16			
16	48	11.91	40.0	1,923	1006	39	3,047	1007	46			
17	41	10.37	38.0	1,320	1011	33	1,863	1009	15	2,380	1011	71
18	49	10.14	7.5	1,939	1009	58	1,910	1009	30	2,367	1008	118
19	40	11.36	36.0	1,213	1020	49	1,613	1014	40	1,846	1014	74
20	20	11.36	41.0	1,201	1019	18	1,097	1017	11	1,400	1013	56
21	48	11.57	38.0	1,250	1021	25	1,031	1013	13	1,533	1021	38
Average						38			23			71

On subjecting these data to analysis the results are found to be statistically significant. Applying the *t* test to the relationship between the control period and the period in which soda bicarbonate was administered we find that  $t = 3.97$  with 40 degrees of freedom which indicates a highly significant difference. Similarly, in comparing the difference between the control period and the period of ammonium chloride administration we find  $t = 3.14$  with 30 degrees of freedom again a statistically significant difference.

*Administration of Sodium Bicarbonate*—It will be noted in the table that without exception, after the administration of this alkaline salt there was a drop in the daily urinary non excretion varying from 04 mg (Patient 8) to 28 mg (Patient 18) per day, or from 15 per cent (Patient 8) to 54 per cent (Patient 12). Analysis of these figures revealed the changes to be statistically significant. The soda bicarbonate produced no consistent or significant change in the amount or specific gravity of the urine. The average daily urinary non excretion for men during the control period was 35 mg and during the period of alkalinization was 22 mg or a decrease of 37 per cent. In the women the average excretion fell from 38 to 23 mg per day or a decrease of 39 per cent.

*Administration of Ammonium Chloride*—This medication was not given to those patients with peptic ulcer but was subsequently administered to the eleven other patients. The urinary non excretion was increased in every case, not only above the period during which soda bicarbonate was given but above the control period as well. In two cases the increase was slight, being 01 and 02 mg respectively, whereas in Patient 18 the increase was 60 milligram. Analysis again revealed these changes to be of statistical significance. In the men the average increase above the control level was 40 per cent, and in the women, 86 per cent. The period of ammonium chloride administration was associated with an increased urine volume in all female patients but in only three of the men.

#### DISCUSSION

These data show that the administration of an alkaline salt diminishes the amount of non excreted in the urine, whereas the administration of an acid salt causes a marked increase when given to patients on a normal dietary non intake. Previous work<sup>13</sup> has shown that achlorhydria decreases the retention of non from a normal dietary non intake, and Mettler and Minot<sup>14</sup> have shown that a better utilization is obtained from small amounts of non when given in an acid medium. Lintzel<sup>15</sup> states that non is absorbed only in an ionized form and that an acid reaction is necessary for this ionization. These data on the urinary non excretion are in keeping with the foregoing observations on the absorption of non from an acid media.

#### CONCLUSIONS

The urinary non excretion from a normal dietary non intake is increased when an acid salt is administered by mouth and decreased when an alkaline salt is given.

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and Dodds, glacial acetic acid was added to the urine hemoglobin mixtures in which turbidity or precipitation had not occurred in forty minutes. Sufficient glacial acetic acid was used to depress the pH from the original levels to a range such as might be encountered during acidosis, approximately a pH of 5. Depending on the combined buffering capacity of urine and the hemoglobin, 1 to 3 drops (0.05 to 0.1 ml) were necessary for each tube. Lowering the pH to 4.8 to 5.2 with acetic acid caused turbidity and precipitation in approximately half of the urine samples. In order to observe the effect of chlorides on hemoglobin solubility in acid urine as well as to determine whether other ions exert any influence, the following tests were carried out: (1) urine samples were analyzed for their ash content by Neumann's method<sup>8</sup>; (2) chlorides were determined by Whitchorn's modification<sup>9</sup>; (3) inorganic phosphorus was determined by the method of Lowenburg<sup>10</sup>; (4) inorganic sulfate by Folio's method<sup>11</sup>; (5) urinary ammonia was determined by Conway's microdiffusion technique.<sup>1</sup> These determinations were done on urine samples which caused the precipitation of hemoglobin without acidification and on ninety-three samples following the addition of acid. In the latter group approximately 50 per cent of the urine samples showed precipitation. Such studies permitted an analysis of the relationship between the concentration of chloride, sulfate, and phosphate anions and the presence or absence of precipitation.

To test the effect of various electrolytes on the solubility of normal and modified hemoglobin experiments were done with solutions consisting of a mixture of a given salt and hemoglobin at pH levels of 4.5 to 5.2. The influence of acidification of hemoglobin previous to mixing with the salts was determined by spectrophotometric absorption at different wave lengths in a Coleman spectrophotometer.

## RESULTS

When urine renders hemoglobin or its derivatives insoluble, one may observe a number of physical changes. Red hemoglobin is converted to a denatured protein, brown in color. The soluble hemoglobin becomes turbid either before or after the color change occurs. The turbidity may persist or in turn, be followed by the formation of visible aggregates which eventually precipitate. Some urine samples will precipitate minimal quantities whereas others can effect a complete precipitation of hemoglobin derivatives. Since the quantity which precipitated was not measured the results are expressed either as positive or negative. Human hemoglobin when added to distilled water and acidified slowly forms a few light colored aggregates without any evidence of turbidity. A comparable reaction was observed rarely in urine and commonly in salt solutions. Hemoglobin in distilled water at a pH of approximately 5.0 was used as a control to ascertain if the test sample caused precipitation.

Following the filtration of urine and mixing with a solution of hemoglobin seven of 302 samples caused precipitation at room temperature within forty minutes. Turbidity without precipitation which we consider as evidence of minimal reaction occurred four times. Therefore eleven of 302 urine samples caused precipitation of hemoglobin derivatives. The pH values in these eleven samples varied from 5.1 to 5.8. In the remaining 291 samples the pH was higher than 5.5, over 90 per cent falling between 5.5 to 6.8.

During these studies it became apparent that temperature exerts a decided influence on the rapidity with which precipitation occurs. To evaluate the influence of temperature sixty-six urine samples were tested simultaneously at room temperature (25 to 31° C) and at 37.5° C. In fifty-nine of

sixty-six samples neither turbidity nor precipitation occurred. In this group the temperature did not exert any apparent influence. In the remaining seven urine samples precipitation occurred once, four revealed turbidity, whereas two were negative at room temperature after forty minutes. Samples of the same urine, when mixed with hemoglobin and placed in the incubator at 37.5° C, all caused precipitation within forty minutes.

The anion concentrations in seven urine samples which caused precipitation at 37.5° C are shown in Table I.

TABLE I. ELECTROLYTE CONCENTRATIONS IN URINE WHICH PRECIPITATED HEMOGLOBIN WITHOUT ACIDIFICATION

PATIENT	DIAGNOSIS	URINE							
		SPECIFIC GRAVITY	pH	ASH (MG %)	$\text{NH}_4^+$ (MEQ / L)	$\text{Cl}^-$ (MEQ / L)	$\text{SO}_4^{2-}$ (MEQ / L)	$\text{H}_2\text{PO}_4^-$ (MEQ / L)	TOTAL ANIONS (MEQ / L)
P. R.	Chronic glomerulonephritis	1.022	5.3	1,154	5.7	41.4	45.0	32.3	118.7
A. J.	Bronchiectasis	1.029	5.2	1,374	5.8	41.2	74.8	37.7	153.7
D. L.	Arteriosclerosis	1.023	5.1	1,030	9.1	34.3	69.0	26.7	130.0
H. M.	Hypertension	1.020	5.2	1,014	5.3	38.1	47.2	20.2	105.6
K. E.	Myxedema	1.020	5.1	1,030	3.4	60.6	50.6	17.4	128.6
C. T.	Ruptured intervertebral disc	1.027	5.4	990	13.1	20.3	62.0	29.3	111.6
C. T.	Ruptured intervertebral disc	1.009	5.8	314	6.7	17.1	14.8	8.7	30.6

The anion and ash concentrations were high in six of seven samples which were analyzed. The pH values varied from 5.1 to 5.8, in six samples the pH was 5.4 or less. Why precipitation occurred in the last urine sample is not apparent, since neither the pH nor the anion concentration is comparable to that of the other six samples. The findings show that hemoglobin is rendered insoluble by urine and suggest that the pH, electrolyte concentration, and temperature exert an effect on hemoglobin solubility.

It seemed desirable at this point to establish whether any of us could excrete urine which was capable of precipitating hemoglobin *in vitro*. Water was restricted to help increase the urine specific gravity. Acid-producing diets, augmented by the oral ingestion of mandelic acid and sodium sulfate separately at spaced intervals, were also taken. It was difficult to depress the urine pH, and the thirst became oppressive. However, after four days on this regime two of five were successful in excreting a urine which did precipitate hemoglobin derivatives within forty minutes at 37.5° C.

Observations on the relationship of urine anion concentration and *in vitro* hemoglobin precipitation following acidification were made in ninety-three samples of urine. The anion concentrations are expressed in milliequivalents per liter of urine. Since the pH was lowered in each case by the addition of acetic acid to a range of 4.8 to 5.2, the ratio of  $\text{H}_2\text{PO}_4^-$  to  $\text{HPO}_4^{2-}$  would be, as is diagrammatically illustrated by Gamble,<sup>13</sup> approximately 50 to 1. For this reason the phosphates are calculated in milliequivalents of  $\text{H}_2\text{PO}_4^-$ . The relationship of precipitation with respect to the various anions at varying concentrations is shown in separate columns in Table II. The range of total

measured anion concentration in single urine samples is also shown. The frequency of precipitation is expressed in fractions, e.g. 3/8 indicates that precipitation occurred in three of eight urine samples at the specified anion concentration.

TABLE II RELATIONSHIP OF IN VITRO HEMOGLOBIN PRECIPITATION FOLLOWING ACIDIFICATION TO THE ANION CONCENTRATION

Cl		SO		H PO		TOTAL ANIONS†	
MEQ/L.	PRECIPITATION	MEQ/L.	PRECIPITATION	MEQ/L.	PRECIPITATION	MEQ/L.	PRECIPITATION
0.20	5/13	0.20	2/38	0.10	7/36	0.25	0/1
21.40	2/14	21.40	10/26	11.20	15/33	26.50	1/6
41.60	5/19	41.00	11/21	21.30	15/18	51.75	0/18
61.80	7/14	61.80	5/5	31.40	4/4	76.100	4/13
81.100	9/15	81.100	3/3	41.50	2/2	101.125	4/17
101.120	7/9					120.150	12/13
121.140	6/7					151.175	14/15
141.60	1/1					176.200	8/8
161.180	1/1					201.225	2/2

Data of the various anions in the first three columns is tabulated on the basis of concentration and not separate urine samples.

†The sum of Cl, SO, and H PO in a given urine sample is shown in the last column.

The relationship of precipitation to individual anion concentration is shown in the first three columns. The chloride concentration was the greatest in most of the samples. Not evident from the table, however, is the fact that the separate anions fluctuated over a wide range independently of each other. This fact explains the presence of precipitation at some of the lowest Cl<sup>-</sup>, SO<sub>4</sub><sup>-</sup>, and H PO<sub>4</sub><sup>-</sup> concentrations. In such urine samples the concentration of the other two anions was usually elevated above average values. The presence of precipitation at the lowest anion concentration in all three columns indicated that this phenomenon is not due solely to the action of a single anion. As the milliequivalent concentration of the different anions increases respectively, the ratio of hemoglobin precipitation also rises.

Examination of the total anion concentration in single urine specimens shows a considerable variation. The range from 51 to 175 meq per liter includes the majority of the urine samples. Precipitation occurred in nine of fifty-five samples in which the total anion concentration was 125 meq per liter or less. In a majority of these which caused precipitation the chloride concentrations tended to be low, whereas the SO<sub>4</sub> values were above average. In thirty-eight samples with a total concentration of 126 meq per liter or more, hemoglobin precipitation occurred in thirty-six samples. It is apparent that when the total anion concentration exceeds 126 meq per liter hemoglobin precipitation almost always occurs at pH values close to 5.0.

A comparison of results in Tables I and II indicates the following. The total concentration of the anions measured in urine samples which caused precipitation without the addition of salts or acids is in agreement with but one exception with the values observed in urine samples following acidification. Interestingly, the SO<sub>4</sub><sup>-</sup> concentrations were higher in the unmodified samples. In some of the urine samples the hemoglobin solubility was not affected as anticipated. That is, precipitation occurred at unusually low total

amon concentrations or in the intermediate range (76 to 125 meq per liter). The cause for hemoglobin precipitation in the unusually low concentrations is not evident. However, when precipitation was observed at concentrations of 76 to 125 meq per liter it was customary to have low  $\text{Cl}^-$  and high  $\text{SO}_4^{2-}$  values.

To determine the influence of different electrolytes on precipitation in a pH range of 4.5 to 5.2, a series of experiments was performed with pure salt solutions and hemoglobin. Solutions of normal and modified hemoglobin were used. The hemoglobin was changed by direct acidification with N/10 HCl until the pH was depressed to 4.92. This acidified hemoglobin was then incubated at 37.5° C for sixty minutes. Prior to mixing with pure salt solutions, the normal and acidified hemoglobin were diluted 1:200 in 0.1 per cent  $\text{Na}_2\text{CO}_3$  and analyzed spectrophotometrically. The absorption curve of the acidified hemoglobin was slightly modified, it crossed the curve of normal hemoglobin at 585 and 512  $\text{m}\mu$ , indicating that acidification and incubation had caused a change in the hemoglobin molecule. Solutions of pure salts at different molar concentrations in 5 ml quantities were mixed with 0.5 ml of hemoglobin, and the pH was adjusted to approximately 5.0, when necessary, with N/10 HCl. The results of some of the studies with mixtures of pure salts and solutions of normal and partially denatured hemoglobin are shown in Table III.

TABLE III THE INFLUENCE OF DIFFERENT SALT CONCENTRATIONS AND THE PURITY OF HEMOGLOBIN ON PRECIPITATION IN THE SPECIFIED pH RANGES

SALT	NORMAL HEMOGLOBIN					ACIDIFIED HEMOGLOBIN				
$\text{NaCl}$	Molar concentration	0.5	0.4	0.3	0.2	0.4	0.3	0.2	0.1	0.05
	pH	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.1
	Reaction	-	-	-	-	+	+	-	-	-
$(\text{NH}_4)_2\text{SO}_4$	Molar concentration	0.4	0.36	0.32	0.28	0.2	0.1	0.05	0.025	0.01
	pH	5.4	5.0	5.0	5.4	5.2	5.2	5.3	4.9	4.9
	Reaction	-	-	-	-	+	+	-	-	-
$\text{Na}_2\text{SO}_4$	Molar concentration	0.5	0.4	0.3	0.2	0.2	0.1	0.05	0.025	0.01
	pH	5.1	5.1	5.2	5.1	5.3	5.2	5.2	5.2	5.2
	Reaction	-	-	-	-	+	+	+	-	-
$\text{NaH}_2\text{PO}_4$	Molar concentration	0.1	0.05	0.038	0.025	0.2	0.1	0.05	0.025	0.01
	pH	5.0	4.9	5.1	5.1	5.1	5.2	5.1	4.9	4.9
	Reaction	+	+	-	-	+	+	-	-	-
$\text{NaH}_2\text{PO}_4$	Molar concentration	0.5	0.4	0.3	0.2	0.5	0.45	0.4	0.35	0.3
	pH	4.7	4.8	4.9	5.0	4.5	4.6	4.6	4.6	4.6
	Reaction	-	-	-	-	+	+	-	-	-

Reactions followed for twenty minutes. Hemoglobin acidified and the pH of salt mixtures adjusted by N/10 HCl acid.

When normal hemoglobin was mixed with solutions of  $\text{NaCl}$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{Na}_2\text{SO}_4$ , and  $\text{NaH}_2\text{PO}_4$  in which the molar concentrations varied from 0.5 to 0.1 precipitation did not occur in twenty minutes. Addition of hemoglobin to  $\text{NaH}_2\text{PO}_4$  solutions in molar concentrations of 0.1 to 0.05 produced minimal precipitation following the adjustment of pH to 4.9 to 5.1. The difference observed when using  $\text{Na}_2\text{HPO}_4$  instead of  $\text{NaH}_2\text{PO}_4$  solutions is probably related to the initial high pH value of  $\text{Na}_2\text{HPO}_4$ , since the final ratio of  $\frac{\text{H}_2\text{PO}_4^-}{\text{HPO}_4^{2-}}$

would be equal after the pH was adjusted. The pI values for  $\text{NaH}_2\text{PO}_4$  are lower than for the rest of the salts because of the difficulty encountered in adjusting the pI to 5.0. The partly modified hemoglobin, on the other hand, was almost completely precipitated within twenty minutes by the higher concentrations of all the electrolytes used.

#### DISCUSSION

Proteins have been shown to be least soluble in the neighborhood of their isoelectric points.<sup>14</sup> In this study, hemoglobin was not precipitated by urine at or near its isoelectric point of 6.4. It is felt therefore that the *in vitro* influence of urine on hemoglobin solubility is not due to this factor. Hemoglobin is known to be amphoteric; it acts as an acid on the alkaline side of its isoelectric point and as a base below a pH of 6.4 when it has a net positive charge.<sup>14</sup> In view of this, one would expect the hemoglobin to bind the anions ( $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ , and  $\text{H}_2\text{PO}_4^-$ ) more strongly at a pH of 5.0 than at its isoelectric point. Furthermore, it is well known that ions of higher valence are more effective precipitating agents. In this respect the  $\text{SO}_4^{2-}$  anion would be quite effective since at a pH of 5.0 it exists almost entirely in the bivalent form.

Green has shown that electrolyte concentration exerts a decided influence on hemoglobin solubility at different pH levels.<sup>15</sup> Green's experiments, however, were conducted at pI levels higher than 5.8. Likewise her studies were done with unmodified hemoglobin whereas the present studies are complicated by changes in hemoglobin and the presence in urine of other solutes which might either augment or inhibit the effect of electrolytes. It is apparent that hemoglobin precipitation in urine *in vitro* is a complex phenomenon in which multiple variables are operating. At the present time the *in vitro* hemoglobin precipitation can be attributed to pI and anion alterations. However, an explanation for the mechanism of precipitation will require further study and would appear to entail individual study of the variables known to exist in this system.

Baker and Dodds<sup>1</sup> originally suggested that two entirely independent factors are required for the precipitation of hemoglobin in urine. First a pH of 5 to 6 is required to convert hemoglobin to methemoglobin and possibly acid hematin. Second, the urine must have a minimal concentration of about 1 per cent NaCl. Our studies are in agreement with the first suggestion that acid urine which converts hemoglobin to other derivatives is conducive to precipitation. However, our findings do not support the hypothesis that a NaCl concentration in excess of 1 per cent is required. In our study the urine samples which caused *in vitro* precipitation of hemoglobin never had a calculated NaCl concentration of 1 per cent. We did, however, find a close relationship between the total anion concentration and precipitation in urine samples in which the pH is 5.4 or less.

It became evident that three factors are necessary before urine causes precipitation of hemoglobin: an acid urine of proper pH, anions in sufficient concentration, and last, a modified hemoglobin. Initially, we were not aware

that hemoglobin modification markedly accelerates precipitation until a study of the influence of pure salts on hemoglobin solubility was undertaken. Baker and Dodds do mention denaturation as a factor in conjunction with acid urine. However, one is not apt to consider hemoglobin changes of significant importance because a pH of 5.0 to 5.4 is desirable in urine before the ions begin to exert significant activity. In view of this, one is likely to assume that hemoglobin modification is merely an associated change rather than a factor which exerts a decided influence on the rapidity and quantity of precipitation.

Whether the ability of urine to precipitate derivatives of hemoglobin is of any importance in the production of hemoglobinuric nephrosis remains to be established. We are able to confirm that acidification of human urine within the physiologic range causes precipitation of modified hemoglobin. It was possible to show that unmodified human urine with the proper acidity and anion concentration also precipitates hemoglobin derivatives. It is hazardous to conclude from *in vitro* experiments whether precipitation will occur *in vivo* under similar conditions. However, one cannot help but wonder whether the mechanisms responsible for hemoglobin precipitation *in vitro* are not also in part responsible for intratubular hemoglobin cast formation.

#### CONCLUSIONS

It has been possible to confirm the observations that acidification of urine within the physiologic range will effect a precipitation of hemoglobin by some of the urine samples.

Urine which is not modified by the addition of either salts or acids and which is capable of precipitating modified hemoglobin can be obtained from some patients. Healthy individuals existing on an acid-producing diet which is augmented by the oral ingestion of mandelic acid and a limitation of water intake are able to excrete urine capable of effecting precipitation of hemoglobin.

Urine which precipitates hemoglobin in most instances would appear to possess several variables. In addition to pH and anion concentration studied in this work it would seem that the known and unknown factors, which are responsible for modification of hemoglobin, play an important role in this system.

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Tyrode solution containing 20 per cent sucrose by volume extracted an average 52.1 mg per cent nonprotein nitrogen in three to four hours by isolated loop perfusion, or more than six times the quantity recovered in any other hypertonic perfusion experiment and ten times the amount recovered with an isotonic solution (Fig 1) The quantity of nonprotein nitrogen extracted in the perfusate was roughly directly proportional to the hypertonicity of the

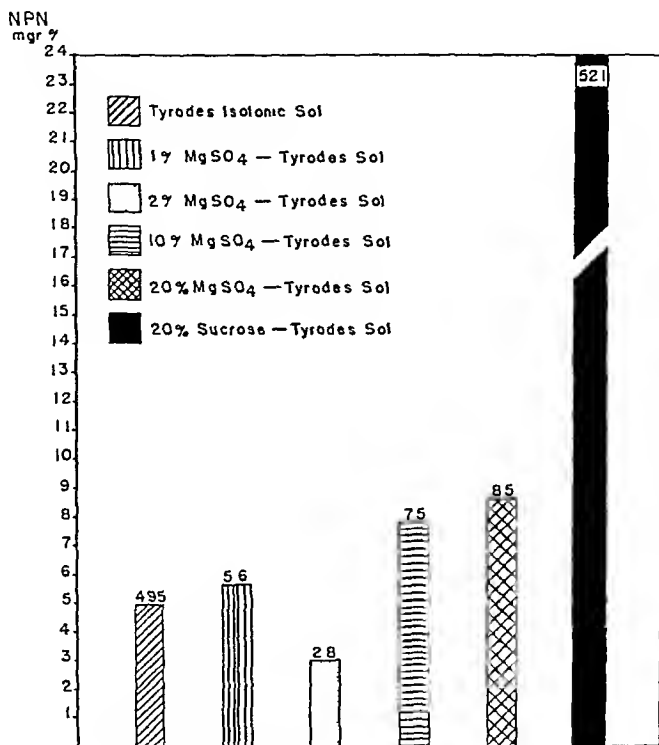


Fig 1—Nonprotein nitrogen recovered in perfusate using Tyrodehypertonic solutions under standard conditions

perfusion solution (Fig 1) Enteric perfusion using Tyrode's isotonic solution produced the greatest reduction of plasma nonprotein nitrogen (Fig 2) No correlation between the degree of plasma nonprotein nitrogen reduction and the amount of nonprotein nitrogen extracted in the perfusate could be established (Figs 1 and 2) Individual variations of protein metabolism, state of hydration, and nonprotein nitrogen-cellular-interstitial space-vascular equilibrium may account for these discrepancies

Tyrode solution containing 20 per cent MgSO<sub>4</sub> reduced plasma nonprotein nitrogen rapidly Dehydration, salivation, vomiting, apparent disorientation, and exhaustion prompted perfusion discontinuance after one and one-half hour respectively Eight hours later, both dogs had fully recovered

Isotonic and hypertonic Tyrode sucrose solutions containing 0.001M HgCl<sub>2</sub> as a protoplasmic poison extracted nonprotein nitrogen in comparable quantity to Tyrode sucrose solutions without the protoplasmic poison (Fig 3)

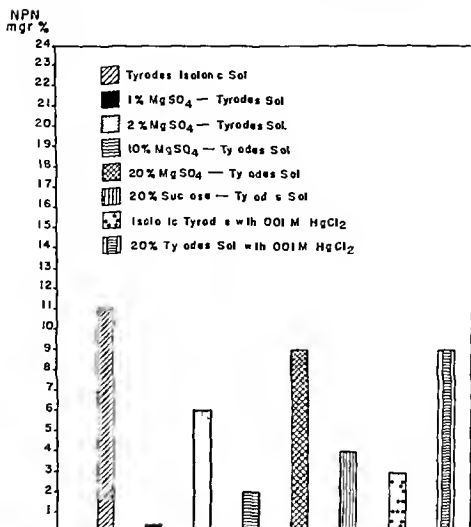


Fig 2—Basal nonprotein nitrogen loss following isolated loop perfusion with hypertonic Tyrode solution under standard conditions

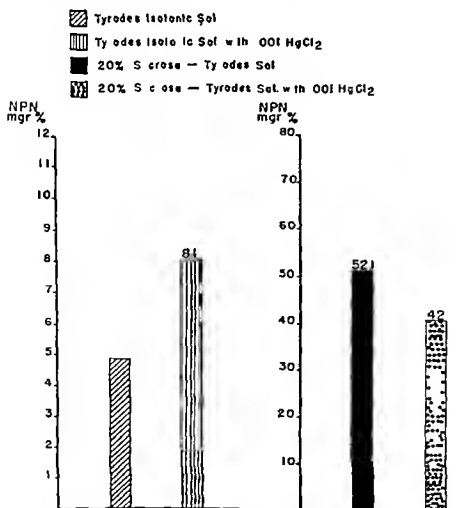


Fig 3—The effect of perfusion fluids containing a protoplasmic poison on the quantity of nonprotein nitrogen recovered (standard conditions)

No constant changes were observed in weight, hematocrit chloride, or total base values following isolated loop perfusion with hypertonic sucrose-Tyiod solution. No general edema nor evidence of hyperchloremic acidosis was encountered during the reported experiments.

Perfusate collected from dogs irrigated with Tyiod hypertonic sucrose fluids gave a negative benzidine occult blood reaction. Microscopic examination showed no significant cellular structures after three to four hours of perfusion.

Increased urine concentration was observed in the majority of experiments, although corresponding weight changes and hematocrit values failed to reflect dehydration. Urine qualitative reduction tests yielded a ++ to +++ reaction in animals perfused with a Tyiod 20 per cent sucrose solution.

#### DISCUSSION

Twenty per cent hypertonic sucrose-Tyiod solution was the most effective perfusion fluid in removing nonprotein nitrogen from the experimental animal. Wells and Johnson<sup>28</sup> have suggested that hypertonic solutions increase the excretion through the bowel wall by producing a chemical or physical irritation rather than by a purely osmotic process. In order that the electrolytic balance of the experimental animal be preserved, and since the process of dialysis is reversible, an irrigating fluid (Tyiod solution) closely resembling the electrolyte pattern of the blood was chosen. Distilled water alone as a perfusion fluid destroys bowel mucosa.<sup>9</sup> Electrolyte balance, particularly with reference to the concentration of the monovalent ions, is not entirely dependent on the action of dialysis alone. Visser<sup>30</sup> has shown that monovalent chloride ions may pass from the bowel lumen by selective action of the mucosa into the circulating blood stream against higher concentration gradients. Comparable clinical observations have been noted by Daugherty and co-workers.<sup>6</sup> Apparent alterations in the electrolyte patterns may be caused by changes in hydration of the experimental animal as well. There was no significant constant change in hemoconcentration as measured by hematocrit values, fluid balance, or electrolyte equilibrium during the perfusion period.

MgSO<sub>4</sub> in concentrations greater than 10 per cent is not satisfactorily tolerated for prolonged periods of perfusion. Greater molecular concentrations of relatively nontoxic molecules in an electrolytically isotonic solution appeared to us to offer a solution for a more effective perfusion fluid. Sucrose was chosen because of its (1) low molecular weight, (2) low toxicity to living tissue, (3) potential caloric value if hydrolyzed and absorbed, (4) availability, and (5) low cost.

With concentrations of sucrose of 50 per cent, vomiting and other toxic manifestations occur. Sucrose in hypertonic solution is damaging to cell structure. It is reasonable to expect that with prolonged irrigation the effect of intestinal perfusion, as measured by the recovery of nonprotein nitrogen might decrease. However, by damaging the action of the mucous membrane with a mercuric chloride 0.001 molar solution<sup>30</sup> in Tyiod solution for perfusion purposes, comparable amounts of nonprotein nitrogen were recovered as compared

with those experiments where isotonic Tyrode solution or 20 per cent sucrose Tyrode solution was used alone. This suggests that the state of physiologic integrity of a bowel wall will not greatly affect dialysis.

Dogs perfused with 20 per cent  $MgSO_4$  Tyrode solution and other high concentrations of hypertonic fluids were able to extract water from the perfusion fluid. In both experiments in which 20 per cent  $MgSO_4$  Tyrode solution was employed as a perfusion fluid, the recovered volume of irrigating solution was less than the volume introduced. Comparable observations have been made by White and Harkins.<sup>9</sup>

## PART II

### MATERIALS AND METHODS

The animals utilized in Part I were bilaterally nephrectomized under sodium pentothal anesthesia. Twenty-four hours after operation, continuous irrigation of the Thiery-Vella loop was instituted with 20 per cent sucrose Tyrode solution modified after the methods employed in Part I. Water but no food was offered during the continuous perfusion period. Intramuscular divided doses of sodium pentothal were administered as a sedative. Parenteral fluids and salts were given intravenously as required based on plasma studies as outlined in Part I.

The following determinations were carried out before nephrectomy and every twenty-four hours thereafter until the animal expired: hematocrit, plasma chloride, total protein, nonprotein nitrogen, and total base. The recovered perfusate was volumetrically measured and examined for urea, nonprotein nitrogen, chlorides, total protein, total base, occult blood, and fragments of tissue. The period of survival was measured from the conclusion of the nephrectomy operation until the animals expired.

### RESULTS

Average survival time of nephrectomized dogs treated by continuous hypertonic (20 per cent sucrose Tyrode solution) isolated loop irrigation was 80.1 hours. In spite of 438 to 612 Gm urea recovered in the twenty-four hour periods, the plasma nonprotein nitrogen progressively increased (Fig. 4). The typical clinical picture of uremia characterized by anorexia, vomiting, excitatory phase, spasmodic muscular contractions, apparent irrationality, lethargy, and death was observed. There was no evidence of edema. All animals lost from 3 to 5 pounds of weight accounted for by (1) vomiting, (2) salivation, (3) insensible water loss, (4) fecal incontinence. Two hundred to five hundred milliliters intravenous fluids per animal per twenty-four hours were sufficient to maintain normal hematocrit values. Total plasma proteins and chloride values remained relatively constant except as temporarily affected by the animal's state of hydration.

Perfusion fluid recovered (24.4 liters per twenty-four hours average volume) yielded 0.2 Gm urea per liter or 4.87 Gm per twenty-four hours (Fig. 4). Occult blood was detected after forty-eight hours of continuous lavage, and fragments of tissue were seen in the perfusate after seventy-two hours.

Post mortem studies revealed no pleural but slight (15 to 20 ml) peritoneal effusion. The Thiery-Vella loops (average length 82 cm) contained multiple mucosal ulcerations from 1 mm to 4 cm in diameter. In two spec-

mens, the isolated loop walls were thickened two to three times normal size by edema and hyperemia. Subserosal hyperemia was pronounced. The amount of bowel damage was directly proportional to the duration of perfusion.

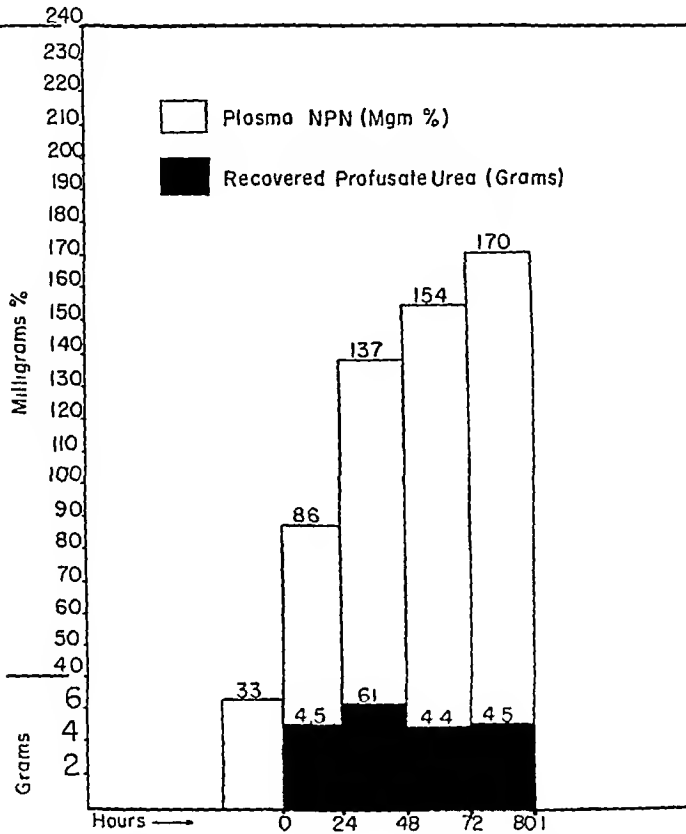


Fig. 4—Relationship of plasma nonprotein nitrogen to recovered urea by continuous hypertonic lavage (20 per cent sucrose-Tyrode solution) in nephrectomized dogs (Average values, five experiments)

### DISCUSSION

Survival time (eighty hours) of nephrectomized dogs treated by continuous hypertonic (20 per cent sucrose-Tyrode solution) perfusion was insignificantly longer than that of the controls of Bliss and co-workers<sup>4</sup> (three days) and White and Harkins<sup>29</sup> (seventy-three hours) and was comparable to that of the controls of Abbott and Shea<sup>1</sup> (three to five days). Survival time of untreated nephrectomized dogs is variable. Ivy, Barry, and co-workers had control animals survive for more than one hundred hours average<sup>3, 10, 18</sup>.

On the basis of comparative survival times of the untreated nephrectomized dogs, any claim of therapeutic success by the method studied must be regarded as invalid.

Hypertonic isolated loop enteric lavage yielded significant quantities of urea in spite of extensive mucosal damage, a fact foreshadowed (Part I) by the

relatively large nonprotein nitrogen yield through the isolated bowel wall, the mucosa of which was damaged by a protoplasmic poison (Fig 3)

It was not within the scope of this work to observe, in great detail, the effect of hypertonic isolated loop lavage upon the chemical structure of the plasma or extracellular fluid. However, from our data, little deleterious effect was observed upon the normal values of proteins, chlorides, total base, and hematocrit. Prolonged continuous irrigation produced mild dehydration readily corrected by intravenous fluid replacement.

We are in accord with Gamble's belief<sup>13</sup> that "in uremia, the defense of the chemical structure of extracellular fluid is of much more importance from the point of view of survival than the reduction of azotemia." Recent work<sup>24</sup> has strengthened this belief.

Therefore, it would seem reasonable that a more promising approach to the therapy of uremia would be a combination of azotemic reduction and proper administration of a balanced solution of electrolytes.

#### SUMMARY AND CONCLUSIONS, PARTS I AND II

1 Hypertonic (20 per cent sucrose Tyrode) perfusion fluid was ten times more effective than isotonic fluid (Tyrode) in extracting nonprotein nitrogen from a dog's isolated enteric loop.

2 The amount of nonprotein nitrogen extracted by isolated enteric lavage was roughly proportional to the hypertonicity of the perfusion fluid.

3 Hypertonic (20 per cent sucrose Tyrode) solution for isolated enteric loop lavage produced no appreciable changes of plasma acid base or water equilibrium over three to four hour periods.

4 Continuous hypertonic (20 per cent sucrose Tyrode) isolated enteric lavage did not prolong the survival period of nephrectomized dogs.

5 Continuous hypertonic (20 per cent sucrose Tyrode) enteric lavage eventually produces mucosal ulcerations and bowel damage.

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# PROGRESSIVE CHANGES IN LIVER COMPOSITION, FUNCTION BODY FLUIDS AND LIVER CYTOLOGY DURING PROTEIN DEPLETION IN THE RAT AND THE EFFECT OF CHOLINE UPON THESE CHANGES

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IT IS known that the liver is the first organ to suffer a serious loss of protein during starvation or the development of protein deficiency. Addis, Poo, and Lew<sup>1,2</sup> found for example that 20 per cent of the protein of the liver of well fed rats was lost during a two day fast and that 40 per cent was lost after a week of fasting. Kosterlitz<sup>3</sup> reports that rats fed a protein free diet lost 15 per cent of the liver cytoplasm during the first day. It thus appears that a considerable amount of the liver protein is easily mobilized. After the first few days the liver losses become much less and according to Campbell and Kosterlitz follow an exponential curve as protein starvation continues.

Cytologic evidence has shown that the ribonucleic acids also are rapidly lost from the liver during protein deficiency. Elman and associates<sup>4,5</sup>, Kosterlitz<sup>3,6</sup> and Wang and Hegsted<sup>8</sup> observed simultaneous clearing and vacuolization of the cytoplasm together with losses of the basophilic staining material which was apparently ribonucleic acid<sup>9,10,11</sup>. It is not clear whether or not these losses represent serious functional changes of the liver.

An accumulation of fat in the liver has been observed during the development of protein deficiency<sup>4,6,12</sup>. In view of the diets which have been used it may be expected that this may be partially related at least to a simultaneous deficiency of choline. Hough and co workers<sup>13</sup> observed that the administration of choline to protein depleted dogs largely prevented the changes in hepatic dye clearance and the rise in serum phosphatase which occurred on the protein deficient diet they were using. It may be expected that the development of protein deficiency in man may also be complicated by low choline intakes.

This paper reports the results of studies of protein deficiency in rats which received diets either poor or rich in choline. Changes in the composition of the liver blood and plasma volumes thiocyanate space bromsulfalein clearance and plasma nitrogen were determined and histologic examinations of the livers were made in an attempt to correlate morphology with the analytic data obtained.

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## EXPERIMENTAL

The study was made with young adult female rats which weighed approximately 200 grams at the time they were given the protein-free diet. The diets were of the purified type previously described<sup>8</sup> and contained glucose, 89 per cent, corn oil, 5 per cent, salt mixture,<sup>14</sup> 4 per cent, cod-liver oil, 2 per cent, and supposedly adequate levels of all of the B complex vitamins except choline in the low choline diet. This diet contained 30 mg of choline chloride per 100 Gm., while the high choline diet contained 500 mg per 100 grams. The animals were housed in individual cages in a room of approximately constant temperature, 78° to 80° F. The diets were fed ad libitum but food intake was recorded at two- or three-day intervals by weighing the amount of food not eaten. Animals were killed at intervals of three, twelve, twenty-five, or more days as indicated below. A total of thirty animals was started upon each diet but it was not possible to make all measurements on each animal. The figures presented below show the number of animals from which data were obtained at each period of deficiency.

At the time of sacrifice, the blood and plasma volumes, thiocyanate space, and biomsulfalein clearance were determined as previously described.<sup>15</sup> The liver was immediately removed, samples were taken from both the right and left lobes for histologic study, and the remainder was prepared for chemical analysis. Water content was determined by drying at 95° C., glycogen and fatty acids plus the unsaponifiable fraction as previously described,<sup>8</sup> and total nitrogen by the macro-Kjeldahl method. Total plasma nitrogen was measured colorimetrically by the Nessler method after digestion.

## RESULTS

*Biochemical Analysis*—Fig. 1 summarizes the changes observed in the liver weight. On the high choline-low protein diet there was an immediate fall during the first three days from approximately 8 grams to about 5.5 grams. This weight was maintained approximately constant for about two weeks and then gradually fell. When expressed as per cent of body weight the original decrement is still apparent, but after a period of depletion it again regained the original relation to body weight and was maintained at this level throughout the depletion. These changes are interpreted as an original loss of liver substance which is much greater than the relative loss of body substances, but during the following two weeks the relative loss of body weight exceeded that of the liver. During the latter stages of depletion the percentage losses from liver and the remainder of the body are approximately equal.

Animals which received the low choline diet failed to show the original liver weight loss because of the accumulation of fat as shown below. During later periods most, but not all, of the fat was lost so that the liver weight changes were approximately the same as in the high choline group. This was also shown by the similarity of the two groups when fat and glycogen were subtracted from the dry liver weight to give nonfat, nonglycogen liver weight, presumably protein weight. This fell gradually and approximately to the same extent in both groups.

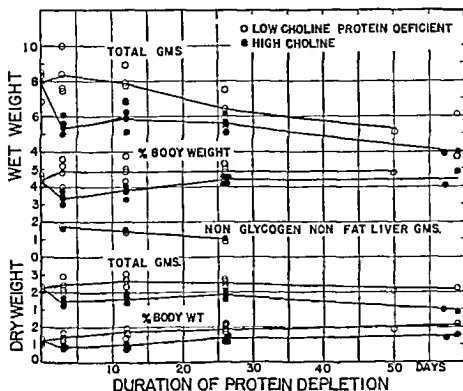


Fig 1—Changes in liver weight with respect to time on protein free diets

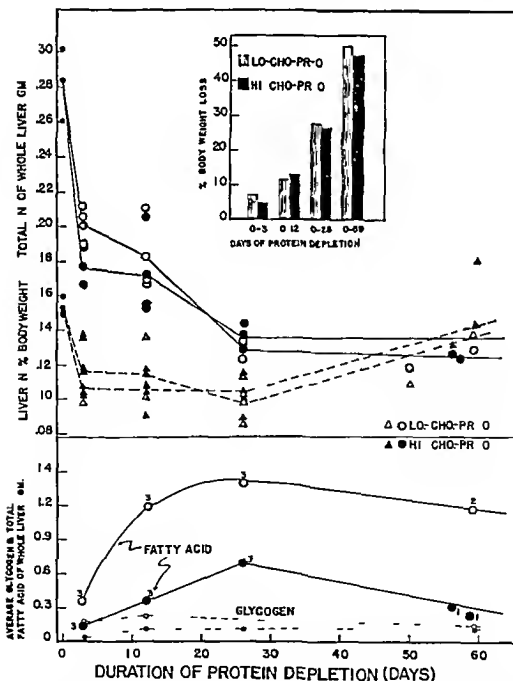


Fig 2—Changes in liver nitrogen, fat and glycogen and body weight losses with respect to time in rats receiving high and low choline, protein free diets. Numbers in the lower part of the figure indicate the number of livers analyzed

The loss of nitrogen from the liver was similar regardless of the choline content of the diet (Fig 2). Body weight loss was also similar in the two groups (Fig 2, insert). In the latter stages of deficiency the rate of body weight loss exceeded that of liver nitrogen loss. Some fat accumulated in the liver during the early stages of deficiency even though choline was supplied. Consistently larger amounts of glycogen were found in the livers of animals which received the lower level of choline.

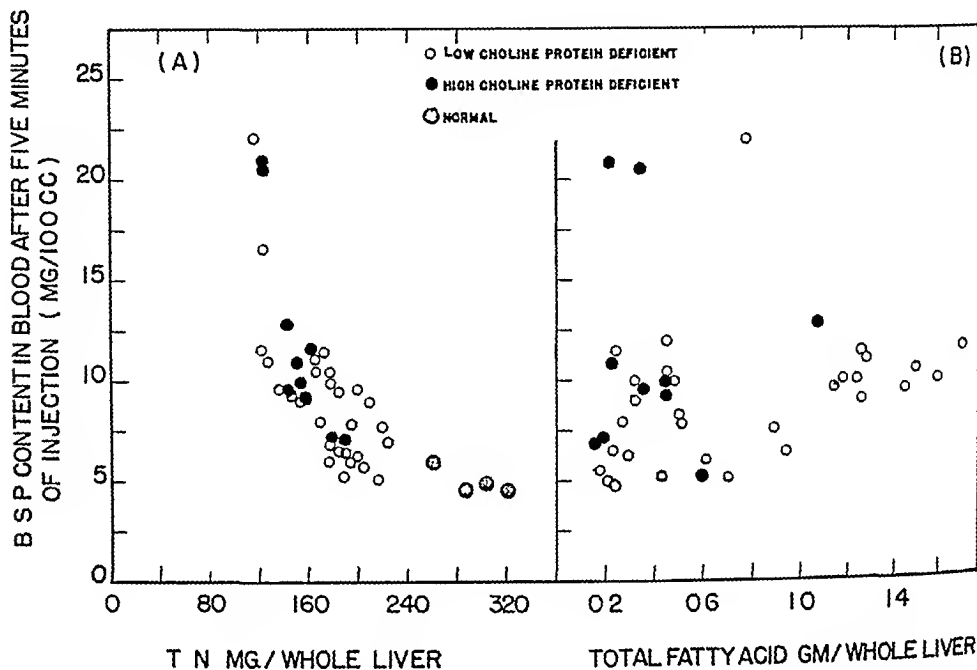


Fig 3—Scatter diagram showing the relation of blood bromsulfalein concentration five minutes after injection to total liver nitrogen as compared with total liver fat. The apparent correlation with liver nitrogen is mainly due to a simple decrease in liver size with loss of weight.

The absolute amount of bromsulfalein cleared from the blood fell markedly during the depletion period. This may be inferred from Fig 3 since the blood concentration of bromsulfalein five minutes after the injection of a 5 mg dose is much higher in those animals with less total nitrogen per liver, that is, the animals subjected to protein deficiency for the longest period of time. It is also clear from the right-hand side of the same figure that the blood level is not well correlated with the degree of fatty infiltration which developed in these animals. Thus as protein deficiency developed, the animals were able to remove smaller amounts of a standard dose of bromsulfalein from the blood in a given time period. However, the deficiency is not only associated with a decrease in liver size but with a fall in body weight and blood volume as well, and an adequate representation of the situation must take these changes into account. Results presented earlier<sup>14</sup> showed that the bromsulfalein clearance of normal rats in the first five minutes after injections yielded a straight-line relationship with dosage.

when both clearance and dosage were expressed in terms of milligrams per 100 grams of body weight. The values obtained with the protein depleted animals were therefore compared with the values expected from normal rats of the same weight. The deviations from normal (negative deviation equals increased bromsulfalein retention) are shown in Table I. In none of the animals was the deviation from the expected value great and none of the mean values presented in Table I were significantly different from the expected value when tested statistically by the 't' test. Thus it is concluded that essentially all the decrease in bromsulfalein clearance is accounted for by the loss of body and liver weight, and there is no proof of malfunction of the remaining liver tissue. It is true that the deviations from normal tend to become greater as the deficiency is prolonged, and additional data might prove these deviations significant statistically.

TABLE I. BROMSULFALCIN CLEARANCE IN PROTEIN DEFICIENT ANIMALS COMPARED WITH VALUES FOR NORMAL RATS

DIET	TIME ON DEFICIENT DIET	NUMBER OF ANIMALS	MEAN DEVIATION OF BROMSULFALCIN CLEARANCE FROM NORMAL (MG/100 GM BODY WEIGHT)
High choline protein free	3	9	-061 ± 024*
	12	10	-149 ± 096
	25	1	-187 ± 095
	40	2	-225 ± 0087
	56	2	-370 ± 322
Low choline protein free	3	7	-076 ± 157
	12	9	-181 ± 137
	24	7	-092 ± 296
	50	1	-40

Standard error of the mean

The changes in the body fluid volumes are indicated in Fig. 4. The thio cyanate space fell gradually and nearly parallel to the fall in body weight so that the ratio of thio cyanate space to total body weight remained constant for approximately forty days. After this period there appeared to be a slight tendency for the thio cyanate space per unit body weight to increase. This increase if significant would represent a tendency toward edema. The changes in plasma volume showed the same trend as those of the thio cyanate space so that the ratio  $\frac{\text{ECFV}}{\text{PV}}$  remained essentially constant even after prolonged depletion.

The total plasma nitrogen concentration showed an early marked drop at the same time that the plasma volume was shrinking. This resulted in a considerable decrease in the total circulating plasma nitrogen (TCPN). The loss in body weight was also large (Fig. 2 insert) and the total circulating plasma nitrogen per 100 grams of body weight showed only a slight and apparently insignificant drop. During the latter stages of the deficiency the plasma nitrogen concentration increased considerably so that the total circulating nitrogen per 100 grams of body weight rose. This occurred simultaneously with the rise in thio cyanate space. It is clear that there was no consistent relation between the plasma nitrogen and the thio cyanate space. Since the formation of edema

is expected to be related to a fall in plasma protein concentration, the changes observed are the opposite of those expected. The total plasma nitrogen figures reflect changes in both plasma proteins and nonprotein nitrogen. Since the

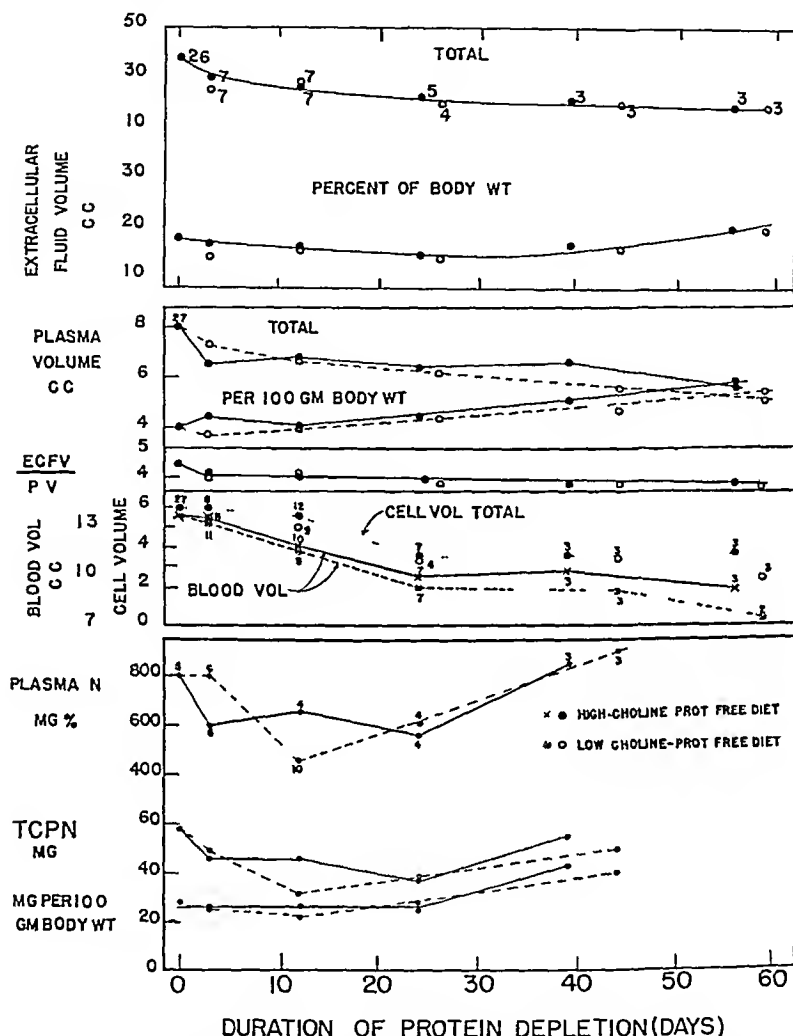


Fig 4—Variations in the distribution of body fluid volumes and plasma nitrogen during protein depletion. Numbers at the various points indicate the number of animals used.

nonprotein nitrogen of typical animals at various stages of depletion remained essentially constant, and less than 30 mg per cent, it is apparent that an increase in plasma nitrogen represents an increase in the total plasma proteins.

None of the data in Fig 4 indicates that choline deficiency and the resultant fatty livers are of importance in the changes in fluid volumes observed.

**Gross Pathology**—As early as the third day, some of the livers appeared paler than those of normal animals. As the deficiency period increased, the livers became successively paler and yellower until at twenty days of deficiency they were definitely and uniformly yellowish-brown rather than the normal

purplish red color. After twelve days on the deficient diet, some animals showed marked involution of the thymus. From this time on, subcutaneous edema appeared in a few of the animals. Although difficult to evaluate because of the great loss of tissue and fat this appeared more severe and more uniform after long periods of deficiency.

*Microscopic Pathology*—Blocks of tissue were fixed in 4 per cent aqueous formaldehyde, Zenker's acetic solution and absolute alcohol. Eosin, methylene blue, hematoxylin and eosin, Best's carmalum, and sudan IV stains were made.

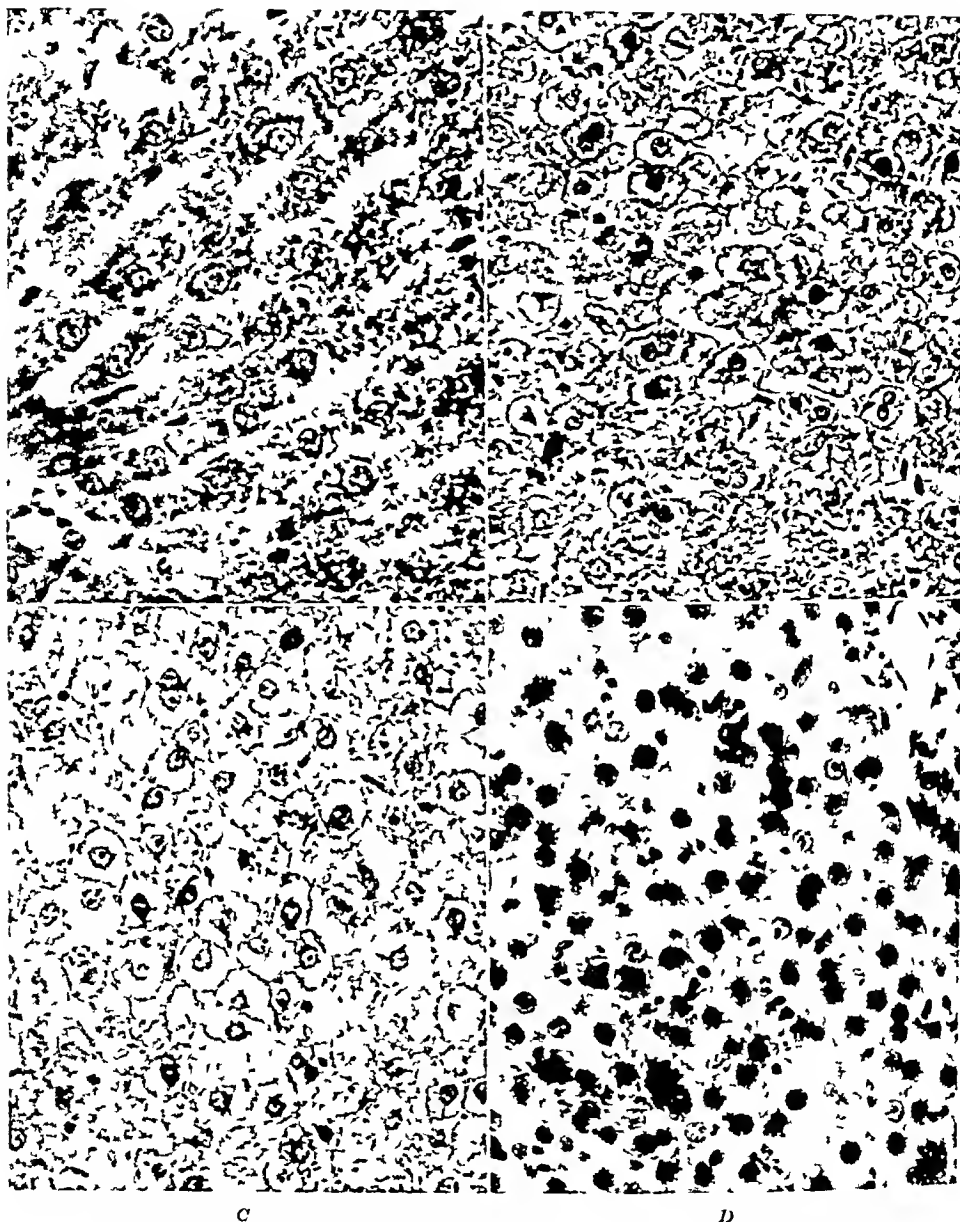
The histologic changes observed were similar to those described by Elman, Smith and Sachar,<sup>7</sup> Wachstein,<sup>10</sup> Kosterlitz,<sup>4</sup> and Kosterlitz and Campbell,<sup>5</sup> and with the exceptions noted below, they were essentially the same in both the high and low choline diets (Fig. 5). The earliest change consisted of enlargement of the liver cells at the expense of the sinusoids, especially in the peripheral zones of the lobules. The sinusoids were compressed and largely obliterated after a few days on the deficient diet. Simultaneously the cytoplasm became less dense ("cytoplasmic clearing") and was represented by a few coarse eosinophilic or basophilic strands. There was an apparent decrease in basophilic granules in the cytoplasm which have been demonstrated to be composed of ribonucleic acid by Caspersson and Schultz and others.<sup>9, 11</sup> The remaining basophilic granules appeared to have been displaced toward the cell membrane which stained deeply and conspicuously ("cytoplasmic rimming").

Whereas in normal animals the nuclei of liver cells were round, vesicular, and uniform in size with finely granular and evenly distributed chromatin, the nuclei of some of the liver cells of rats on the deficient diets were variable in size and shape and tended to be hyperchromatic. These changes were not necessarily more pronounced in those cells showing the cytoplasmic changes described. Also as early as the third day, fat in the form of large or small droplets appeared in the liver cells of the periportal areas. This was more marked in the animals receiving the low choline diet. Best's carmalum stains also indicated an increase in glycogen, mainly in the periportal cells, but the zonal distribution was not as marked as that of fat.

These changes, typical of all the animals on the low protein diets, were more marked in the low choline than in the high choline group. In Table II the various changes have been graded and are listed at different stages of the experiment. In general the most striking changes were consistently observed in animals depleted for twelve to twenty-six days. In a few of the low choline animals, however, cytoplasmic clearing and rimming of equal intensity was found as early as three days. Typically these livers appeared as a mosaic of polygonal clear cells with central nuclei. In the few animals that were sacrificed as late as fifty days it was noted that in the low choline, protein-free group the findings of markedly increased cell size, cytoplasmic clearing and rimming were the same as in the twelve and twenty-six day groups. A moderate amount of fat was found centrally in the lobules, differing from the periportal distribution noted earlier. There was also considerable variation in the size and staining quality of the nuclei. Many cells were multinucleated. In the animals receiving the high choline, low protein diet, the changes were similar except that they

A

B



C

D

Fig. 5—Liver changes with protein depletion. *A* Normal rat (eosin methylene blue  $\times 350$ ). *B* three days of depletion (eosin-methylene blue  $\times 350$ ). *C* twenty-six days of depletion (eosin-methylene blue  $\times 350$ ). *D* seventy-six days of depletion (sud in IV frozen section  $\times 350$ ). As early as three days there is marked rarefaction of the cytoplasm with the remaining basophilic material chiefly at the edges of the cells. The sinusoids have been largely obliterated. At twenty-six days the liver appears as a mosaic of rarefied polygonal cells, but by seventy-six days (high choline protein-free animal) the appearance approaches that found in the well-nourished animal with slight to moderate fatty infiltration chiefly central in location.

became less marked as the depletion was prolonged. A few animals survived as long as seventy-six days and in these the cell size was essentially normal and there was no cytoplasmic clearing or rimming. The fat which persisted tended to be centrally or variably dispersed in the lobules. Nuclei were variable in size and shape.



TABLE II SUMMARY OF THE HISTOPATHOLOGIC CHANGES IN THE LIVER AFTER VARIOUS PERIODS ON THE PROTEIN DEFICIENT DIETS

TIME ON DEFICIENT DIET (DAYS)	CYTOPLASMIC CLEARING		CYTOPLASMIC RIMMING		FAT QUALITY		FAT DISTRIBUTION		NUCLEI		CELL SIZE	
	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE
3	0	++ to +++	0	++ to +++	0 to +	+	Periportal	Periportal	Normal	Normal	0	+
12	+ to ++ (Variable)	++++	0 to +	+++	0 to ++	++	Periportal but some loaded cells throughout	Periportal	Normal or slightly hyperchromatic	Normal	±	+
20	+ to ++ (Variable)	++ to +++ (Variable)	0 to +	++ to +++	+ to ++	++	Periportal	Periportal	Variable in size and stain	Hyperchromatic	±	+
30		+++ (Variable)		++ (Variable)		++		Central		Variable in size and stain		+
60	0		0		+ to ++		Central and variable		Variable in size and stain		0	

0 No change from normal the number of plus signs indicates the relative extent of the changes observed

## DISCUSSION

In general the results of these studies agree with previous investigations<sup>4,6</sup>. The cytologic evidence and the chemical findings both show marked changes in the liver during early protein deficiency. Early in the period of deficiency the large losses of protein are accompanied by a considerable accumulation of fat. The fatty infiltration is apparently not completely preventable by choline in the amounts used, although larger amounts might prove more effective. Glycogen accumulates at the same time and to a greater degree in the livers of the choline deficient animals.

During later periods, when the deficiency is greater, the nitrogen losses from the liver are less and a large proportion of the loss is from the other body tissues. The ratio liver weight/total body weight actually increases at this time. It may be significant that the food intake of the animals is fairly well maintained for three or four weeks upon the diet, but then begins to diminish. During the latter stages of the deficiency there may be a caloric as well as a protein deficiency. The present data are insufficient to evaluate this aspect of the problem, however, since nothing is known of the metabolic rates or caloric requirements of severely deficient animals. It may be expected that the metabolic rate has declined if the animals behave as in starvation, and this together with a marked loss in body weight should lower the caloric needs. However this may be, the evidence is clear that after severe depletion there is a return toward normality in liver structure both chemically and histologically, and this is aided and increased by the presence of choline. There is apparently no basis for deciding whether this is advantageous for the animal, at least from this study. Choline obviously cannot replace protein in the diet, and the animals, regardless of the presence or absence of choline, lose weight, become emaciated, and eventually die. There also does not appear to be any great change in over-all nitrogen metabolism as the result of the degree of fatty infiltration of the liver which occurred on the diets used.

The absolute clearance of bromsulfalein per rat falls markedly during the depletion period, but the clearance per unit body weight is not significantly less than normal. It therefore appears that the total ability of the liver to remove bromsulfalein is greatly changed by the decrease in size, but the function per gram of liver tissue remains nearly normal. This finding is somewhat at variance with the results of previous studies with dogs. In the experience of Hough and Freeman and co-workers,<sup>12, 13</sup> dogs fed a low protein diet soon showed high serum phosphatase values and decreased rates of clearance of rose bengal. However, their basal diet probably contained less choline than our low choline diet. Most of the changes observed by these authors were prevented by the feeding of choline and therefore were due primarily to choline deficiency. McKibbin and associates<sup>17</sup> found lowered bromsulfalein clearance in choline deficient puppies. Similar results also were observed in choline-deficient rats by Maclean, Ridout, and Best,<sup>18</sup> but only in those animals with very large amounts of liver fat. One may conclude that changes in dye clearance per unit body or liver weight due to protein deficiency are relatively slight and are detectable only after relatively severe depletion. On the other hand, unless

choline is specifically added most low protein diets will also be low in choline and this may be the primary factor in lowering the rate of dye removal. The accumulation of fat, however, must be relatively great before it is effective in changing the bromsulfalein clearance in rats.

The modifications in the volumes of the body fluids will receive more attention in a following paper in which the effect of low temperatures has been studied in conjunction with protein deficiency. It may be noted however, that (a) the tendency toward edema as indicated by an increase in thiocyanate space relative to body weight occurred only after very severe protein depletion and was then only slight (b) the plasma volume decreased approximately equally with body weight and the ratio of thiocyanate space to plasma volume remained nearly constant during the depletion and (c) although there was a marked decrease in the plasma nitrogen on the deficient diet, this not only showed no relation to the tendency toward edema but actually was higher at the time when the thiocyanate space tended to increase. This lack of correlation between plasma proteins and edema has been described by Keys and associates in human volunteers subjected to starvation<sup>19</sup> and in several reports of starvation edema from various parts of the world.<sup>20</sup>

It is of some interest that the data, chemical as well as cytologic suggest a return of the liver toward 'normal' as the animal becomes severely depleted. The animal apparently attempts to conserve liver tissue after the first primary loss and as body weight falls the liver weight finally regains its original relation to body weight. Fat is lost from the liver and the composition with regard to the constituents determined in this study becomes nearly normal. Histologically this loss of liver fat is also suggested, as well as an increase in the density of the cytoplasm and an approach toward normal of the appearance of the nuclei cell size, and distribution of the basophilic material in the cytoplasm. It is of course not possible to deduce from these data that the liver has improved functionally.

#### SUMMARY

The progressive changes in liver composition, bromsulfalein clearance, blood and plasma volumes, thiocyanate space, total plasma nitrogen and liver cytology have been followed in rats subjected to severe protein depletion. The effect of low and large supplies of choline upon those changes has been studied.

In agreement with the findings of previous investigators the liver shows a very rapid loss of nitrogen while fat and glycogen accumulate. The fat accumulation is only partially preventable with choline in the amounts used. Cytologically the livers show a rapid loss of basophilic material, the cytoplasm becomes less dense and the sinusoids are largely obliterated. These changes are progressive for approximately one month. After more prolonged depletion the liver size approaches its original relation to body weight, the amount of fat and glycogen decrease and the liver approaches the original cytologic state.

The fall in plasma volume and thiocyanate space during the first month of depletion was approximately proportional to the decrease in body weight. After prolonged depletion the volume occupied by these fluids per unit body weight increased slightly and proportionately. The total circulating plasma nitrogen

per 100 grams of body weight was also essentially constant for approximately one month and then increased slightly

Although the bromsulfalein clearance per rat decreased markedly during protein depletion, this change was accounted for by the loss in weight which occurred. No proof of malfunction of the remaining liver tissue was obtained by this test.

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## BROMSULFALEIN CLEARANCE

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THE conventional bromsulfalein test has been widely recognized as one of the better determinations of liver function<sup>1,2</sup> although it does not detect minor degrees of impairment. Furthermore it has been noted that in some instances the results of this method do not coincide either with clinical evaluation or with other tests. It was hoped that by determining the rate of clearance of bromsulfalein from the blood stream rather than by measuring retention alone a more sensitive index of liver function could be obtained.

This report deals with an improved bromsulfalein test and an evaluation of liver damage in patients with chronic gall bladder disease as measured by this test.

### METHOD

The subjects used in the study consisted of a control group of forty-two patients with no known liver pathology submitted to elective hemorrhaphy, another group of thirty-eight patients submitted to elective cholecystectomy, and a third miscellaneous group of eighteen subjects with known severe liver damage including patients with hepatitis, cirrhosis and malignant metastases to the liver.

The standard 5 per cent bromsulfalein solution was used and all tests were run by multiple determinations with the patients under basal conditions. For this purpose an indwelling needle attached to a glass adapter and a rubber cap<sup>3</sup> was inserted into an arm vein and left in place. A sample of blood was drawn as a control before the injection of the dye and others were obtained at intervals during the test. Small amounts of diluted heparin solution (1000 IU per cubic centimeter diluted to 1 in 50 with normal saline) were injected through the rubber cap between samples to keep the needle open. The dye was administered intravenously into the other arm at a slow rate in order to prevent the irritating effect of the concentrated material from producing a phlebitis or if extravascular a severe cellulitis. Care was also necessary in the sampling to prevent hemolysis. All samples were immediately centrifuged without anticoagulant with a portable centrifuge.

The serum was treated by a procedure modified for use with the Coleman Junior spectrophotometer Model 6 A. One half cubic centimeter of serum was mixed with 2 c.c. of 1 per cent saline and the mixture was divided into two equal parts. One portion was acidified by the addition of 0.05 c.c. of acidified 1 per cent HCl made by adding 1 c.c. of concentrated HCl to 100 c.c. of 1 per cent NaCl. The other portion was alkalinized by the addition of 0.05 c.c. of 5 per cent NaOH.

For each blood sample including the control the spectrophotometer was set at 100 (no dye) at wave length 580 with the acidified portion then the alkalinized portion was read. In this way a correction was made for any hemolysis in each specimen and for any substances in the control serum other than bromsulfalein which might affect the reading.

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Originally the readings were made on a photometer but later the more accurate spectrophotometer was used exclusively. The block comparator method of estimating retention was found too gross.

Spectrophotometric readings were converted to plasma concentration in milligrams per cent from a scale made previously with known dilutions of the dye

With regard to dosage, subjects were divided into three groups, multiple samples being obtained in all. In one, single tests were performed, in another, different doses of dye were used on the same individual, and in the third, repeated tests were run on the same patient before and after surgery, using the same quantity in each instance. The dose used in this last group was determined preoperatively by giving variable amounts on successive days, in order to obtain a dose which would give levels high enough to be read accurately.

All results were graphed on semilogarithmic paper, using the abscissa for time and the ordinate for dye concentration. In this way the rate of removal of the dye was readily visualized. The clearance coefficient,\* which represents the slope of such a graph, was used to express a numerical result of the test. The coefficients used in this study were for the ten minute period from twenty to thirty minutes following injection of dye. The reasons for using this period are given in the discussion.

## RESULTS

*Types of Clearance Obtained With Multiple Samples*—Twenty reliable tests were obtained by this method. With a normal circulation time, there is a rapid rise in concentration to a peak at one to three minutes after injection, because of mixing (Fig 1). Thereafter there is a rapid fall which, in patients with good liver function, may continue until disappearance of the dye. In the presence of poor liver function, there is a slowing in the rate of removal starting at approximately fifteen to twenty minutes and continuing until all the dye has disappeared (Fig 2).

*Normal Value for Clearance Coefficient*—It was found that there was great variation in the values of clearance coefficient in the different groups. No detectable change in plasma dye level in the ten-minute period from twenty to thirty minutes following dye injection gives a coefficient of zero. This result is obtained in cases of obstructive jaundice and the test is therefore not used in them. The group of patients with known liver damage all showed very slow excretion and coefficients less than 0.037. The gall bladder group showed better liver function, average coefficient for this group was 0.063. But a few in this group had severe liver damage, as shown by the lowest reading obtained (Table I), 0.003. The control group (hernias) had repeated preoperative tests. A clearance coefficient of 0.037 was established on the controls as the lower limit of normal (90 per cent of the group had values higher than this). Because the average for this group was 0.082, values ranging from 0.08 to 0.03 should be considered as border line and below 0.03 as definite evidence of liver damage.

*Effect of Variation in Dosage*—This group of tests was made in order to determine the role of dosage in the rate of clearance in the same patient. Altogether twenty-five reliable comparisons were obtained in this group, which

\*The clearance coefficient for any time interval during dye removal was approximated by the following formula

$$k = \frac{\log_e [B_1] - \log_e [B_2]}{t_2 - t_1}$$

where k = the first approximation of the clearance coefficient.  $B_1$  = bromsulfalein concentration at ( $t_1$ ) time of collecting first sample and  $B_2$  = bromsulfalein concentration at ( $t_2$ ) time of collecting second sample.

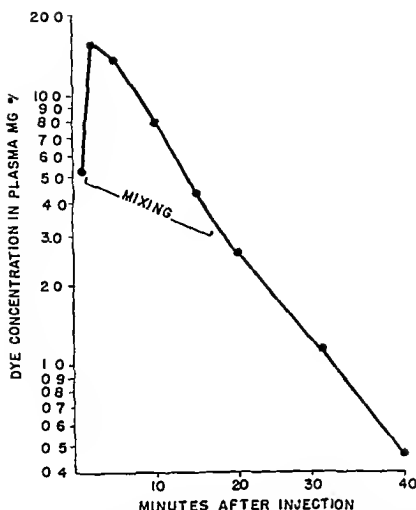


Fig 1—Bromsulfalein clearance in patient with good liver function. Mixing shown in early part of graph (All graphs on semilogarithmic paper)

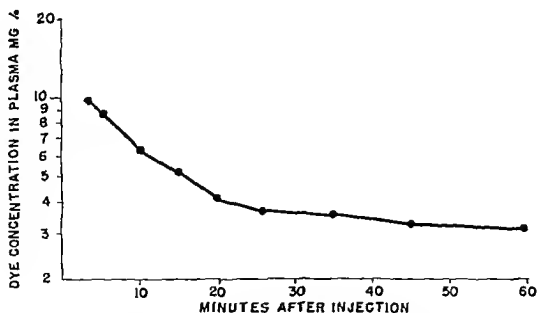


Fig 2—Bromsulfalein clearance in patient with poor liver function. Slow prolonged excretion of dye

included patients with normal and abnormal liver function. These tests were obtained under basal conditions and at such times in the clinical course that significant changes in liver function would not be expected. In most cases a 5 mg per kilogram dose was given first then depending on the concentrations attained, the next dose was increased or decreased.

TABLE I RESULTS OF PREOPERATIVE BROMSULFALEIN TEST EXPRESSED AS CLEARANCE COEFFICIENT IN PERIOD FROM 20 TO 30 MINUTES AFTER INJECTION

OPERATION	NUMBER OF CASES	RANGE	AVERAGE
Cholecystectomy	38	0.140-0.003	0.063
Herniorrhaphy	42	0.168-0.022	0.082
Difference			0.019*

\*Difference is statistically significant

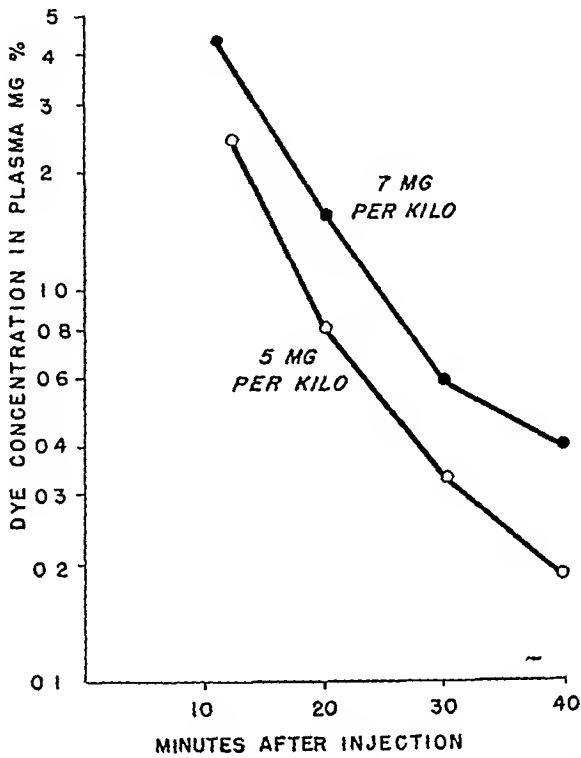


Fig 3 --Patient with good liver function. Same clearance in spite of change in dye dose on consecutive days

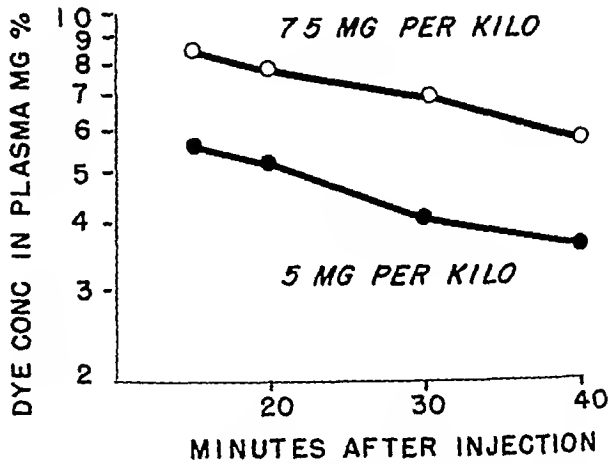


Fig 4 --Patient with cirrhosis. Identical clearance in spite of change in dye dose on consecutive days



Figs 3 and 4 illustrate the rate of clearance in two patients following different dosage given on different days. The concentration of dye following a dose of 7 or 7.5 mg was greater than that following a 5 mg dose, but the rate of clearance was essentially the same. This is true in the patient with cirrhosis (Fig 4), as well as in the one with a normal liver (Fig 3). This parallelism in the rate of excretion was noted in all such tests conducted on other patients.

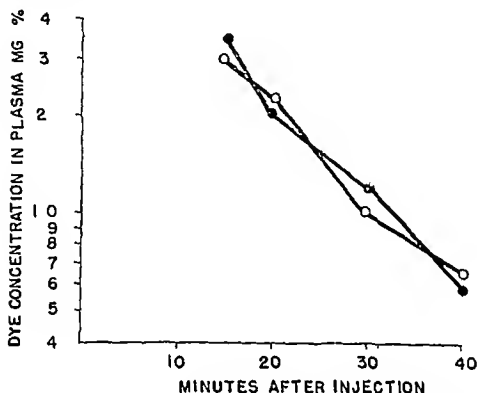


Fig 5—Duplication of excretion graph. Same dye dose on consecutive days

*Changes in the Clearance Coefficient Produced by Operative Load*—In patients on a fixed regimen and in a stable physiologic state repetition of the test on consecutive days revealed identical curves for rate of clearance (Fig. 5). However, following hemorrhaphy or cholecystectomy (under gas ether anesthesia) changes were observed. In eight individuals the rate of clearance was followed preoperatively and every day after the surgical procedure until the preoperative clearance was reestablished. In most instances a test also was done on the afternoon of the day of surgery.

Following operation in every instance there was a lowering of the clearance coefficient. The greatest depression of clearance appeared on the afternoon of the day of surgery. The return to normal in most instances took four to five days. High fever, wound hematoma and other complications were immediately reflected by a slowing in the rate of clearance of the dye. The patients showing the best clearance before surgery demonstrated the least slowing as a result of the operation and recovered earliest.

*Value of Clearance Coefficient in Determining the Minimal Liver Damage in Patients With Chronic Cholecystitis*—The results of the clearance coefficient of bromsulfalein in patients admitted for elective cholecystectomy were compared with those obtained from a group of normal subjects (hemorrhaphy). This was done to determine if the test would demonstrate liver damage in patients with gall bladder disease. As shown in Table I, the gall bladder group had a

lower coefficient of clearance as compared with the control group. The difference is statistically significant ( $P = 0.015$ ). Since the gall bladder group consisted of older individuals than the control group, it was thought desirable to determine whether the difference between the two was not merely due to age.

TABLE II AGE DISTRIBUTION

OPERATION	CASES	AVERAGE AGE	UNDER 40	OVER 40
Cholecystectomy	38	42.63	13 (34.21%)	25 (65.79%)
Herniorrhaphy	42	36.46	26 (61.90%)	16 (38.10%)

It can be seen from Tables II and III that when grouped according to age alone no difference was apparent, but that a difference still existed between the gall bladder and the control groups. The control patients under 40 years of age revealed a higher coefficient than those in the same age group with gall bladder disease, the same is true for those over 40.

TABLE III RELATIONSHIP OF CLEARANCE COEFFICIENT OF BROMSULFALEIN TO AGE  
(RESULTS ARE THE AVERAGE SCORE FOR THE GROUP)

OPERATION	UNDER 40	OVER 40	ALL AGES
Cholecystectomy	0.059	0.066	0.063
Herniorrhaphy	0.080	0.085	0.082

## DISCUSSION

The customary method of performing the bromsulphalein test is dependent upon the assumptions that the patient's blood volume is 10 per cent of the body weight and that furthermore plasma volume is proportional to body weight. It has been considered that patients receiving the same dose of dye per kilogram body weight have the same plasma concentration of dye at the start of the test. This assumption appears to be a basic source of inaccuracy. It is obvious that, in persons of asthenic habitus or with changing fluid balance because of disease or other cause, plasma volume may be far from constant with relation to body weight. Patients with cirrhosis of the liver best typify this error. A large part of their body weight may be ascitic fluid and in them the dose of dye will be abnormally great per unit of plasma volume. Because of such observations, this work was designed to investigate the manner of removal of the dye from the blood and, if possible, to find a better way of measuring its removal.

As noted earlier, in patients with poor liver function, a flattening in the slope of dye excretion was noted fifteen to twenty minutes after injection. This phenomenon has been referred to by others and has led to speculation as to its meaning.<sup>11-13</sup> The liver is the main site of removal of this dye from the blood,<sup>3, 13</sup> while other tissues account for small amounts, especially the extrahepatic reticulo-endothelial system including the spleen.<sup>5, 8</sup> Rosenthal, some years ago, demonstrated that the dye was adsorbed to protein and suggested that the size of this combination molecule accounted for its nonappearance in the urine of normal patients. After certain *in vitro* experiments,<sup>12</sup> he also proposed that the action of bile salts on surface tension allowed liberation of the dye from plasma protein in the liver. More recent work of Blauer and Pessotti<sup>2</sup> is in line with Rosenthal's theory and assigns to the true albumin fraction of human plasma the role

of binding bromsulfalein. The latter workers propose an exchange of protein bound dye between plasma and hepatic cells as the basic mechanism. This theory of removal from plasma seems acceptable. Retention of dye following India ink injection<sup>4, 8, 11</sup> may be just a mechanical blocking of the Kupffer cells with resulting decreased surface area for this physical reaction to occur.

With the usual dose of 2 or 5 mg per kilogram of body weight, the blood is cleared of the dye in from thirty to forty five minutes<sup>10</sup> but it keeps appearing in the bile for at least two hours and in some instances for as long as five or six hours<sup>3, 13</sup>. Excretion from the liver into bile is therefore obviously a separate mechanism. In patients with liver disease appearance in the bile is delayed and disappearance from the bile is slow. It seems reasonable then to assume that in these cases slow removal from the blood after fifteen or twenty minutes is a reflection of excretion from the liver into the bile. This is in contrast to the first fifteen or twenty minutes which measures mainly transfer from plasma to liver and so represents only one phase of the mechanism. This assumption would explain the fact that in nearly every patient, normal or abnormal, early fall in blood levels is relatively rapid, but the slow removal in liver disease is the most noticeable in the later stage of the test. If the liver can dispose of the dye rapidly it may continue to remove it from the blood at its maximum rate of acquisition and so in the normal subject no flattening of the graph occurs. However, if excretion into the bile is slow, then the liver becomes blocked and hence can lower the blood level later only at this reduced rate of clearance into the bile. This biphasic mechanism then, is one reason why the rate of excretion after twenty minutes can be considered more indicative of actual liver function than the response before this time.

The choice of thirty minutes as the late limit of the determinations is based on the principle of decreasing reliability of readings with high dilutions. At forty minutes there is often little or no dye left with the 5 mg dose. Therefore accurately timed samples at twenty and thirty minutes have been taken and the clearance coefficient obtained for this time interval has been considered as an index of the efficiency of the removal mechanism (mainly liver). If greater accuracy were required three samples could be taken at twenty, twenty five, and thirty minutes after injection, the coefficient from twenty to twenty five and from twenty five to thirty minutes calculated and the average determined. Thus without prolonging the test any error in one specimen could be minimized.

It may not be amiss to call attention to the work of Blumberg and Schloss<sup>1</sup> who have shown that circulatory inadequacy will slow dye excretion. Thus poor clearance in a cardiac patient with congestive failure may be evidence of poor circulation alone or combined with liver damage.

#### SUMMARY AND CONCLUSIONS

The results obtained with a modification of the bromsulfalein test, utilizing the clearance coefficient are presented. For practical purposes, the coefficient during the interval from twenty to thirty minutes after dye injection was found to be most useful, a lower limit of normal for this period being determined

This procedure measures the actual manner of removal of the dye and is a better index of liver function than present methods of studying dye retention. With it a comparison was made between a group of subjects with normal liver function and patients who were candidates for elective cholecystectomy and hence expected to show liver damage, and a significant difference was demonstrated between the two types of subjects.

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## THE CONCENTRATION OF COMPONENT A IN BLOOD, ITS ASSAY AND RELATION TO THE LABILE FACTOR

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FOR clinical purposes it is advantageous to divide the clotting factors required for the formation of thrombin into two categories—the thromboplastin forms and constituents of the prothrombin complex.<sup>1</sup> The latter are essential for prothrombin activity as measured by the one stage prothrombin time. According to Quick and associates the agents in the complex are the labile factor, components A and B, and bound calcium.<sup>2, 3</sup> The labile factor is characterized by its instability especially in decalcified plasma and its nonadsorbability by tin calcium phosphate. Component A is the principle which is adsorbable by tin calcium phosphate, requires vitamin K for its synthesis and is deficient in one type of congenital hypoprothrombinemia. It is probably this component which is generally called prothrombin by other investigators. Component B is difficult to define. Its existence is postulated to explain a second type of congenital hypoprothrombinemia, in which the labile factor and component A appear to be normal.<sup>4</sup> This type of hypoprothrombinemia is particularly important because it is hereditary and the lowered fixed prothrombin time may run through several generations. Little is known concerning the functions of calcium in the formation of thrombin, but recent studies clearly show that it is not ionic but bound calcium that participates in the reaction.

The desirability of having methods for the quantitative estimation of the various constituents of the prothrombin complex is obvious. Recently we developed a simple procedure to estimate the concentration of the labile factor and found that rabbit blood contains fifty times and dog blood ten times as much as human blood.<sup>5</sup> In the present report experiments are described which not only yield a procedure for the estimation of component A but also furnish results which show its relationship to the labile factor.

### METHODS AND MATERIAL

The one stage method for the prothrombin time procedure was employed exactly as outlined by its originator.<sup>7</sup> The thromboplastin used was acetone dehydrated rabbit brain. It uniformly yielded a prothrombin time of 11 to 12 seconds for normal human plasma.

The tricalcium phosphate treated plasma was prepared according to directions previously described.<sup>6</sup> Plasma thus prepared is free of component A but retains practically all of its fibrinogen and all of the labile factor. Care was taken to take only the minimum quantity of tricalcium phosphate required, i.e., an amount just sufficient to render the plasma incogulable to excess thromboplastin and an optimum concentration of calcium.

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Each lot of stock solution was tested on the oxalated plasmas of the various species employed in the study. All studies were carried out on oxalated plasma (1 vol of 0.1M sodium oxalate to 9 vol of blood). It should be emphasized that tricalcium phosphate does not remove component A from citrated plasma.<sup>5</sup> For convenience, plasma treated with tricalcium phosphate is named in this paper either "calcium phosphate plasma" or " $\text{Ca}_3(\text{PO}_4)_2$  plasma."

*Isolation of a Concentrate of Component A*—The tricalcium phosphate after its removal from the plasma by centrifugation was washed with water and again packed by centrifuging. The water was poured off and the last traces adhering to the sides of the tube were removed with a filter paper. For every cubic centimeter of plasma treated, 0.1 cc of 0.2M sodium citrate was added and the mixture stirred for ten minutes. The tricalcium phosphate was removed by centrifugation. The eluate contained approximately 90 per cent or more of the component A present in the plasma.

*Vitamin K Deficiency*—Newly hatched chicks were put on a vitamin K free diet and the procedure was followed as recently outlined.<sup>2</sup>

## RESULTS

*The Estimation of the Concentration of Component A in Plasma by Mixing With Plasma Devoid of This Factor*—Since complete avitaminosis K can be achieved readily in chicks, a plasma showing little or no prothrombin activity was easily obtained. When such a plasma was progressively diluted with normal hen plasma, the prothrombin time decreased in accordance with the typical hyperbolic curve until the 50 per cent dilution was reached. After this the prothrombin time remained the same as that of the normal hen plasma (Table I).

TABLE I THE EFFECT ON THE PROTHROMBIN TIME WHEN AVITAMINOTIC K CHICK PLASMA AND HEN PLASMA TREATED WITH TRICALCIUM PHOSPHATE ARE PROGRESSIVELY DILUTED WITH NORMAL HEN PLASMA

Plasma of vitamin K deficient chick (cc)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.0
Normal hen plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Prothrombin time (sec)	$\infty$	16	14	13½	12	11½	11	11	11	11	11
Hen plasma treated with $\text{Ca}_3(\text{PO}_4)_2$ (cc)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.0
Normal hen plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Prothrombin time (sec)	$\infty$	17	15	14	12	11½	11	11	11	11	11

Exactly similar results were obtained when normal hen plasma was diluted with hen plasma from which component A was adsorbed with tricalcium phosphate. These results clearly show that the concentration of component A in normal hen plasma can be reduced almost 50 per cent before the prothrombin time begins to increase. In other words component A may vary between 50 and 100 per cent without affecting the prothrombin time. This was strikingly illustrated by injecting vitamin  $\text{K}_1$  into a chick with an initial prothrombin time of 10 minutes. Four hours later it was reduced to 11 seconds, i.e., to the same level as that of a hen on a complete diet. However, on comparing the prothrombin times obtained when this and normal hen plasma were mixed with plasma free of component A, it is plain that the concentration of component A in the chick blood was definitely lower (Table II). If one uses the data of Table I as a guide, it can be concluded that 0.03 cc of hen plasma contains as much component A

as 0.05 cc of the chick plasma, yet both had a normal prothrombin time. It is clear that component A does not become the determinant of the prothrombin time until its concentration is reduced below 50 per cent of the normal level.

TABLE II THE CONCENTRATION OF COMPONENT A IN THE BLOOD OF A VITAMIN K DEFICIENT CHICK AFTER THE ADMINISTRATION OF VITAMIN K<sub>1</sub>

Prothrombin time of chick plasma before giving vitamin K		10 min	
Prothrombin time of chick plasma 4 hr after giving 10 γ of vitamin K <sub>1</sub>		11 sec	
Vitamin K deficient chick plasma	0.05 cc	Vitamin K deficient chick plasma	0.05 cc
Normal hen plasma	0.05 cc	Chick plasma after vitamin K	0.05 cc
Prothrombin time (sec.)	11½	Prothrombin time (sec.)	13½
Hen plasma treated with Ca <sub>3</sub> (PO) <sub>4</sub>	0.05 cc	Hen plasma treated with Ca <sub>3</sub> (PO) <sub>4</sub>	0.05 cc
Normal hen plasma	0.05 cc	Chick plasma after vitamin K <sub>1</sub>	0.05 cc
Prothrombin time (sec.)	11½	Prothrombin time (sec.)	13½
Normal hen plasma	0.05 cc	Chick plasma after vitamin K	0.05 cc
Saline	0.05 cc	Saline	0.05 cc
Prothrombin time (sec.)	14½	Prothrombin time (sec.)	15

Because of the difficulty of attaining severe vitamin K deficiency hypoprothrombinemia in mammals and the relative infrequency of avitaminosis K in man, it was not possible to run a dilution titration such as was made with chick plasma. One of us (A. J. Q.) however found in his records of 1938 two important experiments. The first was the determination of the effect of mixing the plasma of a newborn infant having a prothrombin time of 45 seconds with an equal volume of normal human plasma. A prothrombin time of 12 seconds was obtained. Similarly, when the plasma of a jaundiced patient (prothrombin time 48 seconds) was mixed with normal human plasma the resulting prothrombin time was 12½ seconds. In both plasmas component A was greatly diminished, yet on adding half the volume of normal plasma the prothrombin time was restored to normal even though the mixture contained only about one half as much component A as normal plasma.

By using fresh human plasma treated with tricalcium phosphate as diluent the concentration of component A in normal plasma can be determined (Table III). Interestingly, almost identical results were obtained when rabbit plasma adsorbed with calcium phosphate was employed as diluent, provided the human plasma was collected with the utmost care to preserve platelets. It will be observed that as the calcium phosphate plasma was mixed with increasing amounts of normal plasma the prothrombin time decreased until a dilution of 50 per cent was attained. Beyond this the prothrombin time remained the same as that of normal plasma. As in the case of hen plasma, the prothrombin time remained normal until the concentration of component A was reduced below 50 per cent of the amount present in normal blood. Again it is clear that the concentration of component A is not the determinant of the prothrombin time. The labile factor likewise is not the deciding factor. Rabbit plasma contains fifty times as much labile factor as human plasma yet the results with rabbit and human calcium phosphate treated plasmas as diluents are almost identical.

TABLE III THE EFFECT ON THE PROTHROMBIN TIME WHEN NORMAL HUMAN PLASMA IS PROGRESSIVELY DILUTED WITH HUMAN AND WITH RABBIT PLASMA FROM WHICH COMPONENT A WAS REMOVED BY ADSORPTION WITH TRICALCIUM PHOSPHATE

Normal human plasma (cc)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.00
Normal human $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10
Prothrombin time (sec.)	12	12	12	12	12	12	13	15	18	26	$\infty$
Normal human plasma (cc)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.00
Rabbit $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10
Prothrombin time (sec.)	12	11½	11½	11½	11½	12	13	15	18	40	$\infty$

\*The blood was collected in a silicone-coated syringe and transferred to a silicone coated test tube containing sodium oxalate.

*Congenital Hypoprotrombinemia Deficiency of Component A*—It was fortunate that in the pursuit of this problem two patients with congenital hypoprotrombinemia of the type that lacks component A were available. Studies of the blood of these two brothers were reported previously.<sup>4</sup> Characteristically, when 1 volume of their plasma was mixed with an equal volume of normal human plasma, a normal prothrombin time was attained. In fact when the hypoprotrombinemic plasma was progressively diluted with normal plasma, a curve was obtained (Table IV) which was almost identical with that observed when avitaminotic K chick plasma was diluted with normal hen plasma. This is easily explained by postulating that both the chick's and the patient's plasma lacked component A, and since normal plasma contains nearly twice as much as is needed to produce the normal fixed prothrombin time, this minimum value is obtained when 50 per cent of the volume of normal plasma is added to the defective plasma. Interestingly, when the deficient plasma is diluted with normal human plasma treated with tricalcium phosphate, the prothrombin time increases progressively with the dilution and is practically identical with the results obtained when saline is the diluent. When rabbit plasma treated with tricalcium phosphate is used, the prothrombin times again increase progressively, but the values are shorter than those obtained with human calcium phosphate plasma. An explanation for this finding requires further study. It is clear that the plasmas of the two boys which have a consistent prothrombin time of 19 to 21 seconds lack component A. According to Quick's original prothrombin curve, those values indicate a prothrombin activity of 25 to 30 per cent of normal. In the light of the present findings, the concentration of component A in terms of per cent of the concentration of A in normal plasma may actually be lower than 25 per cent.

The present findings as well as other results from recent studies carried out in our laboratory show that the prothrombin time as determined by the one stage procedure does not measure any particular constituent of the prothrombin complex but rather the composite effect. The true determinant of the relatively fixed prothrombin time of 12 seconds in normal human plasma still remains unexplained. Because of the low concentration of the labile factor, after twenty



TABLE IV THE EFFECT OF THE PROTHROMBIN TIME WHEN PLASMA FROM A PATIENT WITH CONGENITAL HYPOTHROMBINEMIA (DEFICIENCY OF COMPONENT A) IS DILUTED WITH HUMAN AND WITH RABBIT PLASMA ADSORBED WITH  $\text{Ca}_3(\text{PO}_4)_2$  AND WITH SALINE

Plasma from patient (cc)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Normal human plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Prothrombin time (sec)	21	14	13	12	11½	11	11	11	11	11
Plasma from patient (cc)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Human $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Prothrombin time (sec)	21	23	24	25½	27	38	55	70	29	600
Plasma from patient (cc)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Rabbit $\text{Ca}(\text{PO})$ plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Prothrombin time (sec)	21	19½	19½	20½	21	22	23½	24½	35	53
Plasma from patient (cc)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03		
Saline solution	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07		
Prothrombin time (sec)	21	24	25	27	31	39	50	75		

The patient was a 4 year old boy. He and his brother age 7½ years had prothrombin times which ranged between 19 and 22 seconds. Both had a mild bleeding tendency.

four hour storage human plasma may show a decrease of 50 per cent of prothrombin activity caused by destruction of this agent whereas rabbit plasma may show no loss as determined by the prothrombin time for a week or longer because the labile factor is in great excess. Component A may be decreased 50 per cent, according to the result of this study before the prothrombin time is prolonged. These findings do not condemn the one stage method, but rather emphasize that it measures the over all prothrombin activity. For the determination of the specific factors methods must be devised which are basically modifications of the one stage procedure. Thus a simple method of the assay of the labile factor was developed in our laboratory in which stored human plasma is the testing medium.\* From the results of the present study a simple determination of component A can be outlined. A sample of fresh oxalated plasma to be tested is mixed with increasing volumes of fresh normal human plasma treated with tricalcium phosphate and the prothrombin times of the mixtures are determined. If the concentration of component A is normal the results will be identical with those given in Table III. By means of eluting with sodium citrate the tricalcium phosphate to which component A is adsorbed a new means for assaying this component becomes available as will now be described.

*The Concentration of Component A in Human and in Dog Plasma as Determined by Re-adding to Component 1 Free Plasma the Factor Concentrated by Adsorption and Elution*—In this study evidence was offered to show that tricalcium phosphate removes only component A from oxalated plasma, therefore plasma thus treated becomes an ideal assay medium for determining component A. All that is required is to add it to the calcium phosphate plasma and determine the prothrombin time. Since the only significant component adsorbed is A and since sodium citrate elutes it almost completely the eluate is presumably a relatively pure and concentrated solution of component A. Since 0.1 cc of sodium citrate is employed to elute the product adsorbed from 1 cc of plasma, this amount when re-added to 1 cc of calcium phosphate treated plasma should restore the prothrombin time to normal. This was found to be true if human

eluate was added to human plasma, and dog eluate to its respective plasma, but when human eluate was added to dog plasma ( $\text{Ca}_3(\text{PO}_4)_2$  treated) or rabbit plasma somewhat shorter times were obtained. On the other hand, when dog eluate was added to human plasma, the resulting prothrombin time was approximately the same as that of normal human plasma. In Table V the prothrombin times obtained by adding component A eluate in progressively decreasing amounts to deprothrombinized plasma are presented. It will be observed that by adding decreasing amounts of human eluate to human deprothrombinized

TABLE V THE QUANTITATIVE EFFECT ON THE PROTHROMBIN TIME OF ADDING COMPONENT A (CONCENTRATED BY ADSORPTION AND ELUTION) TO HUMAN, DOG, AND RABBIT PLASMAS TREATED WITH  $\text{Ca}_3(\text{PO}_4)_2$

Normal human $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from normal human plasma (cc)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	12	12	12½	13½	14	15	17	21	28	45
Expected value from Quick's prothrombin curve	12	12	12½	13½	14	15	17	19	25	34
Rabbit $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from normal human plasma (cc)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	10½	10½	11	11	11½	12	13¼	14	19	31
Normal human $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from dog plasma (cc)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	10	10	10½	11	11¼	11½	12	12	12½	19
Dog $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from dog plasma (cc)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	6	6	6	6	6	6	6¼	6¾	7	8½

plasma, prothrombin time values are obtained which correspond closely to the values of Quick's prothrombin curve. It also should be noted that only one third to one-fifth as much dog eluate as human eluate must be added to human deprothrombinized plasma to obtain a normal prothrombin time of 12 seconds. This clearly shows that dog plasma contains several times as much component A as is normally present in human plasma. This agrees with the original findings obtained with the one-stage method that dog plasma contains five times as much prothrombin as human plasma<sup>10</sup> and casts serious doubt on the hypothesis that the difference between the prothrombin times of dog and human plasma is due to variations in conversion factors.

The reason dog eluate added to human depiothrombinized plasma produces a prothrombin time of only 10 seconds instead of 6 seconds as in dog plasma is the inadequate concentration of the labile factor in human blood. Either component A or the labile factor can be the limiting factor in the production of thrombin and in determining the prothrombin time. This is strikingly illustrated by Experiment I. A small quantity of rabbit plasma supplied sufficient labile factor to reduce the prothrombin time to 10 seconds. This emphasizes the importance of controlling the concentration of the labile factor in assaying component A. Fortunately, the concentration of the labile factor has been found to have a fixed and relatively constant level for any particular species. When human plasma is employed as the assaying medium it should be used within a few hours after the blood is collected and be preserved in an ice bath. Normal human plasma does not contain sufficient labile factor to permit its use for assaying concentrated solutions of eluate such as is obtained from dog plasma.

## EXPERIMENT I

Stored human Ca (PO) <sub>4</sub> plasma	0.09 cc	Fresh human Ca (PO) <sub>4</sub> plasma	0.09 cc
Fluate from human plasma	0.01 cc	Fluate from human plasma	0.01 cc
Prothrombin time	25 sec	Prothrombin time	12 cc
Stored human Ca (PO) <sub>4</sub> plasma	0.08 cc		
Rabbit Ca (PO) <sub>4</sub> plasma	0.01 cc		
Eluate from human plasma	0.01 cc		
Prothrombin time	10 sec		
Prothrombin time of stored plasma	25 sec.		

*The Concentration of Component A in the Plasma of Congenital Hypoprotrombinemia*—The lack of component A in the type of congenital hypoprotrombinemia studied is clearly shown by the results given in Experiment II. The prolonged prothrombin time of Subject B B is due purely to a deficiency of component A. The eluate obtained from his plasma when tested for component A potencies either on normal calcium phosphate plasma or on his own plasma yielded the same low result whereas the eluate from normal human plasma restored his prothrombin level to normal exactly as it did in normal calcium phosphate plasma.

## EXPERIMENT II

Subject B B	Prothrombin time	20 sec	(Congenital hypoprotrombinemia)
Normal subject	Prothrombin time	11½ sec	
B B Ca <sub>3</sub> (PO) <sub>4</sub> plasma	0.09 cc	B B Ca (PO) <sub>4</sub> plasma	0.09 cc
Eluate from B B plasma	0.01 cc	Eluate from normal human plasma	0.01 cc
Prothrombin time	29 sec	Prothrombin time	11½ sec
Normal human Ca (PO) <sub>4</sub> plasma	0.09 cc	Normal human Ca <sub>3</sub> (PO) <sub>4</sub> plasma	0.09 cc
Eluate from B B plasma	0.01 cc	Eluate from normal human plasma	0.01 cc
Prothrombin time	25 sec	Prothrombin time	11½ cc

*The Concentration of Component A in Rickets*—After chicks were on a vitamin K free diet for ten days a severe hypoprotrombinemia was produced in fact a prothrombin time greater than 8 minutes occurred in about one fourth of a group of twenty chicks. An eluate of the plasma of these chicks

showed no prothrombin potency when tested on hen calcium phosphate plasma, indicating a complete lack of component A. An eluate obtained from normal hen plasma when tested on the plasma of these avitaminotic K chicks immediately restored the prothrombin to the same level as this amount of eluate did in normal hen plasma treated with tricalcium phosphate, Experiment III. It is logical to conclude that deficiency of vitamin K produces a pure component A type of hypoprothrombinemia.

## EXPERIMENT III

Prothrombin time of normal hen plasma	11 sec
Prothrombin time of vitamin K deficient Chick 1	480 sec
Prothrombin time of vitamin K deficient Chick 2	600 sec
Prothrombin time of vitamin K deficient Chick 3	Infinity

Hen $\text{Ca}_3(\text{PO}_4)_2$ plasma	0.09 cc	Chick 1 plasma*	0.09 cc
Eluate from hen plasma	0.01 cc	Eluate from hen plasma	0.01 cc
Prothrombin time	13 sec	Prothrombin time	13½ sec

\*The prothrombin times of the plasmas of Chicks 2 and 3 after the addition of the eluate of normal hen plasma were 12½ and 13 seconds respectively. Previous treatment of these plasmas with tricalcium phosphate did not change their response to eluate.

*Concentration of Component A in Plasma After Administration of Dicumarol*—From the work in our laboratory, evidence has been obtained that the hypoprothrombinemia caused by Dicumarol is not identical with that brought about by vitamin K deficiency. Recently Dam and Sondergaard<sup>11</sup> have also come to the conclusion that the two types of hypoprothrombinemia are not identical, although they emphasize that the primary deficiency in both is the "classical prothrombin."

In the present investigation, no attempt was made to determine the basic difference between the two types of hypoprothrombinemia. The sole objectives were to ascertain the quantitative effect Dicumarol had on the concentration of component A and to determine the response of Dicumarol plasma to the addition of an eluate of component A obtained from normal plasma. It was found, as shown in Table VI, that the response of Dicumarol plasma to the eluate from dog plasma was the same as that of normal plasma. This shows that the hypoprothrombinemia resulting from Dicumarol can be promptly and completely corrected by the addition of a concentrated solution of component A, i.e., the eluate from the tricalcium phosphate adsorbed plasma.

TABLE VI THE RESPONSE OF NORMAL AND DICUMAROL PLASMA TO THE ADDING OF COMPONENT A OBTAINED AS AN ELUATE FROM NORMAL AND DICUMAROL PLASMA

Normal dog $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.098	0.096	0.094	0.092	0.09
Eluate from normal dog plasma (cc)	0.002	0.004	0.006	0.008	0.01
Prothrombin time (sec)	16	8	7	6½	6
Dicumarol dog $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.098	0.096	0.094	0.092	0.09
Eluate from Dicumarol plasma (cc)	0.002	0.004	0.006	0.008	0.01
Prothrombin time (sec)	16	8	7	6½	6½
Normal dog $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.09	Dicumarol dog $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0.09		
Eluate from Dicumarol plasma (cc) *	0.01	Eluate from Dicumarol plasma (cc) *	0.01		
Prothrombin time (sec)	38	Prothrombin time (sec)	67		

\*Eluate obtained from Dicumarol plasma which had a prothrombin time of 48 seconds.

From the results recorded in Experiment IV it is evident that this correction can be effected not only *in vitro* but *in vivo* as well. One can readily comprehend the therapeutic implications of this experiment. By two simple procedures, namely, adsorption with tricalcium phosphate and elution with sodium citrate one can concentrate the quantity of component A contained in 500 cc of blood to 25 cc and have it immediately suitable for intravenous injection. The clinical evaluation of the eluate is being studied at present.\*

## EXPERIMENT IV

Dog F, weight 8 kg given 5 mg of Dicumarol per kg of body weight daily for 4 days	
Eluate (concentrated component A) prepared from 22 cc. of fresh normal dog plasma	
Prothrombin time before injection of eluate	30 sec
Prothrombin time after injection of eluate	16 sec.

The basic defect in Dicumarol poisoning is lack of component A, the same as in avitaminosis K. This makes the hypothesis made by one of us (A J Q),<sup>2</sup> that Dicumarol causes a decrease of component B, untenable. Nevertheless there is evidence that the hypoprothrombinemia due to lack of vitamin K is not identical with that due to Dicumarol. It will be noted that the eluate obtained from Dicumarol plasma is more effective in normal than in Dicumarol plasma. Studies are being carried out to determine whether this observation is significant.

## SUMMARY

When normal ovalated plasma is added in progressively increasing amounts to plasma from which component A of prothrombin is absent or very low due either to vitamin K deficiency removal by adsorption with tricalcium phosphate or congenital lack the prothrombin time is characteristically decreased until approximately 1 volume is added to 1 volume of the defective plasma. After this the prothrombin times become the same as that of the normal plasma. This indicates that normal plasma supplies about twice as much component A as is required to attain a normal prothrombin time and that therefore it is apparently not the specific determinant of the prothrombin time even in the presence of an excess of the labile factor.

By means of adsorbing ovalated plasma with tricalcium phosphate and treating the latter with sodium citrate, an eluate is obtained which contains almost all of the component A present in the plasma. When this eluate is added in an amount equivalent to the volume of normal plasma from which it was obtained to plasma lacking component A, the normal prothrombin time is restored. By adding progressively decreasing amounts of the eluate to component A free plasma a series of values of prothrombin time are obtained which correspond well with the original prothrombin curve. Component A is active only if an adequate amount of the labile factor is present. The isolation of component A as an eluate and the testing of it on fresh tricalcium phosphate treated plasma offer a new method for its quantitative estimation.

The therapeutic possibilities of employing the eluate in various types of component A deficiencies are discussed. Its efficacy in reducing the prothrombin time in Dicumarol poisoning is illustrated in dogs.

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# INTENSIVE IMMUNIZATION OF AN ALREADY SENSITIZED Rh NEGATIVE WOMAN, BIRTH OF A MILDLY DISEASED BABY

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**O**NCE a woman has given birth to an Rh positive child with a fatal form of erythroblastosis, it is very uncommon for her to give birth to one less diseased.<sup>1 2 3 4</sup> This is still more rare when the woman has been sensitized by both pregnancy and transfusion. When the donor of the transfusion is the husband the outlook is almost hopeless. This situation developed in the family here recorded. The record was as follows:

## Family Blood Groups —

Dr E N E (husband) Group 0 MN P positive S negative CDe cDe (R<sub>1</sub>R<sub>0</sub>)

Mrs E N E (wife) Group 0, MN P positive S negative, cde cde (r<sub>1</sub>)  
N J E (son) Group 0 S negative CDe cde (R<sub>1</sub> r)

Mr C E (father of Dr E N E) Group 0 S negative, CDe cde (R<sub>1</sub> r)

Mrs C E (mother of Dr E N E) Group A<sub>1</sub> cDE cDe (R<sub>1</sub> R<sub>0</sub>)

Mrs N C (sister of Dr E N E) CDe cDe (R<sub>1</sub>R<sub>0</sub>) ‡

## Pregnancy Record of Mrs E N E white age 35 years —

October, 1940 Abortion at twelve weeks followed by hemorrhage and pneumonia. Transfusion of 500 cc of husband's blood without reaction.

May 1942 Delivery of living child (N J E) moderately jaundiced for one week.

August 1945 Spontaneous abortion at eight weeks.

August, 1946 Delivery of macerated fetus at thirty nine weeks. Active fetal movements ceased two weeks prior to delivery. A necropsy was performed on the fetus §. The following are the gross findings:

Fetus weight 3500 gm length 48 cm. Very marked edema over the whole body, especially occiput, buttocks, and extremities. Numerous ruptured blebs were present leaving a raw surface. Lips and tongue were purplish black in color. There was no fluid in the pleural or pericardial cavities. The lungs were brown firm and on section like cut meat. The heart and GI tract were normal. The liver was enlarged and of purplish brown color. The spleen was also enlarged.

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‡

	C	D	I	c	e	C*
Dr E. N. E.	+	+		+		-
Mrs E. N. E.	-			+		
N J E.			-	+		
Mr C E.	+		-			
Mrs C E.		+		+	+	-
Mrs N C.	+	+	-	+		-

These tests were performed by the Vancouver Red Cross Laboratory Vancouver B.C. Canada and by Dr R. K. Race Fleiter Institute London England

§By Dr R. J. D. McNally Pathologist Royal Jubilee Hospital Victoria B.C. Canada

TABLE I

DATE	AMOUNT OF INJECTIONS (C C)	Rh ANTIBODY TITERS			SUBJECTIVE REACTIONS	NOTES
		SALINE ANTI C	ALBUMIN ANTI C	ALBUMIN ANTI D		
Intravenous						
1947 Jan 14	2	0	0	1 64	0	
Jan 21	3				0	
Jan 28	4 5	1 2	0	1 512	+	
Feb 4	6				+	Benzidine test urine—trace
Feb 11	8				+	
Feb 18	9	1 8	0	1 130,000	0	
Feb 26	10 5	1 8	0	1 16,000,000*	0	
Mar 5	13	1 8	0	1 1,028	++	
Mar 12	15	1 16	0	1 4,000	0	
Mar 19	17 5	1 4	0	1 16,000	++	
Mar 25	19 5	1 8	0	1 4,000	+++	
Apr 2	23 5	1 8	0	1 2,000	++	
Apr 8	27				+++	
Apr 16	30	1 8	0	1 4,000	++	
Apr 23	33	1 8	0	1 8,000	0	
May 1	37	1 8	0	1 1,000	++	
May 7	40	1 16	0	1 1,000	+	Serum albumin— 4 8 Gm
May 14	43	1 16	0	1 1,000	0	Serum globulin— 3 3 Gm
May 21	46	1 16	0	1 1,000	+	
May 29	50	1 16	0	1 512	++++	
June 4	53	1 8	0	1 512	+++	Urine benzi dine—+
June 11	53	1 2	0	1 512	+++	Urine benzi dine—+
June 18	54	1 2	0	1 256	++	Urine benzi dine—++
June 25	56	1 2	0	1 128	++++	
July 2	50	1 2	0	1 128	++++	Icteric index—10, at height of reaction
July 9	35	1 2	0	1 256	++++	
July 16	20	1 2	0	1 256	++++	Last menstrual period (beginning of pregnancy)
July 25	0	Trace	1 8	1 128		
Aug 22	5				++++	
Intramuscular						
Sept 1	5				0	
Sept 6	8				0	
Sept 11	8	Trace	1 16	1 128	0	
Sept 16	8				0	
Sept 21	8				0	
Sept 26	8	0	0	1 128	0	
Oct 1	8				0	
Oct 6	8				0	
Oct 10	8				0	
Oct 15	8				0	
Oct 20	8				0	
Oct 25	8				0	
Oct 30	8	0	0	1 128	0	
Nov 4	8				0	
Nov 9	8				0	
Nov 14	8				0	
Nov 19	8				0	
Nov 24	8				0	
Nov 29	8				0	
Dec 4	8	0	0	1 128	0	
Dec 9	8				0	



TABLE I—CONT D

DATE	AMOUNT OF INJECTIONS (CC)	Rh ANTIBODY TITERS			SUBJECTIVE REACTIONS	NOTES
		SALINE ANTI C	ALBUMIN ANTI C	ALBUMIN ANTI D		
Dec 14	8				0	
Dec 19	8				0	
					0	
Dec 24	8	0	0	1 512		23rd week of pregnancy
Dec 29	8				0	
1948 Jan. 3	8				0	
Jan 8	8				0	
Jan 13	8	0	0	1 512	0	
Jan 18	8				0	
Jan 23	8				0	
Jan 28	8				0	
Feb 2	8				0	
Feb 7	8				0	
Feb 12	8				0	
Feb 17	8				0	
Feb 22	8				0	
Feb 27	8	0	0	1 512	0	
Mar 3	8				0	
Mar 8	8				0	
Mar 13	8				0	
Mar 18	8	0	0	1 1 000	0	
Mar 23	8				0	
Mar 29	0	0	0	1 4,000		Delivery at 37th week
Oct 29		0	0	1 512		Seven months post partum

Data on Rh isoimmunized woman (Mrs E. N. E.) showing injections intravenously and intramuscularly of husband's Rh positive blood. The Rh antibody titers are shown. (Tests performed by the Vancouver Red Cross Laboratory Vancouver British Columbia and occasionally confirmed by Dr Philip Levine Ortho Research Foundation Raritan N. J.) Throughout the titrations an absent or weak agglutination (negative prozone) to the albumin (blocking) anti D factor was noted in the first two to five tubes. Subjective reactions of the patient were graded as follows:

- + Slight, transient shooting pains usually of fingers
  - ++ Few chills joint pains (see +) headache for one hour
  - +++ More severe chills pains and headache lasting for two hours
  - ++++ Chills joint pains backache severe headache temperature to 101 F. Reaction lasting about three hours but responding in part to antihistaminic drugs
- Tests for hemoglobin in the urine (benzidine test) were negative except as noted. Tests for urinary urobilinogen were consistently negative.

It would seem unlikely that this titer of 1 16 000 000 obtained on Feb 6 1947 is correct. The test was not confirmed.

The kidneys, adrenals and pancreas were normal. The occipital bones were both fractured in several places. The brain showed no evidence of injury or hemorrhage but marked softening and edema were present. The placenta was hypertrophic, pale and edematous.

Microscopic sections were prepared and reviewed\*. The report follows:

Single sections of tissue fixed in formalin and stained with hematoxylin and eosin were available from the following tissues: lung, adrenal, spleen, placenta, diaphragm, liver, thymus and kidney. Sections of the liver and spleen were stained for iron. Lung: The alveoli were distended with amniotic fluid but there was no inflammatory reaction. Normoblasts and large erythroblasts were present in large numbers in the capillaries of the alveoli and also in some of the larger vessels. There were several islands of blood formation in the subpleural connective tissue. These were chiefly erythropoietic but there was also

some leucopoiesis. *Adrenal* Radial streaks of cells of the fetal cortex contained each a single large vacuole which looks like a fat droplet. In some places in the adult cortex the capillaries are well preserved and contain many nucleated red cells, both normoblasts and erythroblasts. *Spleen* There was very extensive autolysis but cell nuclei could be seen throughout the section. No lymphoid follicles could be seen. There was an abnormal amount of iron within cells in clots and streaks as one sees the iron in erythroblastosis. *Placenta* Most of the villi were small with a condensed stroma but about 20 per cent were larger with a loose, empty and possibly edematous stroma. The maternal sinusoids were empty. The capillaries of the villi were fairly well preserved and contained nucleated cells, both normoblasts and erythroblasts. The remaining organs added no information, autolysis being far advanced. From the above we can say (1) The fetus had attempted to breathe in utero, aspirating amniotic fluid. (2) There had been an abnormal degree of blood destruction with deposition of iron in excess in the spleen. (3) There was abnormal blood formation with immaturity of the red cell series in the circulating blood. From these it can be concluded that the baby almost certainly had erythroblastosis and it can be assumed that the degree of anemia was so great as to cause anoxia with resulting attempted respiration. Finally, the lesion in the adrenal is commonly seen in fetuses and newborn infants dying of erythroblastosis, particularly those with hydrops, but also sometimes occurring in other clinical forms of the disease. I do not think it possible to say for certain that the fetus suffered from hydrops.

On the basis of gross and microscopic findings a diagnosis of erythroblastosis seems justified and it is assumed that death was due to this disease. A confirming factor was the persistence of Rh antibody to a titer of 1:256 (albumin anti-D) in the mother's blood two months post partum.

The outlook for the birth of a normal child to this couple appeared hopeless. It was therefore decided to attempt to "desensitize" or "stabilize" the woman by serial, increasing injections intravenously of her husband's blood. The possibility of severe reactions was recognized as well as the probability of making this isosensitized woman even more sensitive. The program of the procedure is presented in Table I and Fig. 1.

By the twenty-fourth injection (June 25, 1947) a maximum intravenous injection of 56 c.c. of the husband's blood was reached and it was obvious that no further increase could be undertaken without danger to the patient. Reactions proved almost as severe with the lesser amounts of 35 and 20 cubic centimeters. The patient had reached a stage of extremely marked sensitivity. In fact, she was given 5 c.c. of blood intravenously and suffered a reaction almost as severe as with 56 c.c. previously and much greater than with 43 c.c. on the way up. At this time it became apparent that the patient was pregnant, her last menstrual period having been on July 14, 1947. It was accordingly decided to give the husband's Rh-positive blood intramuscularly instead of intravenously. There were no subjective reactions to the intramuscular blood. The patient received a total of twenty-eight intravenous and forty-three intramuscular injections. From Nov. 9, 1947 (sixteenth week of pregnancy) until delivery the patient was given 3 to 5 Gm. per day orally of methionine (Meonine\*).

\*Meonine brand of methionine. Kindly supplied by Wyeth & Bro. Ltd., Walkerville, Ontario, Canada.

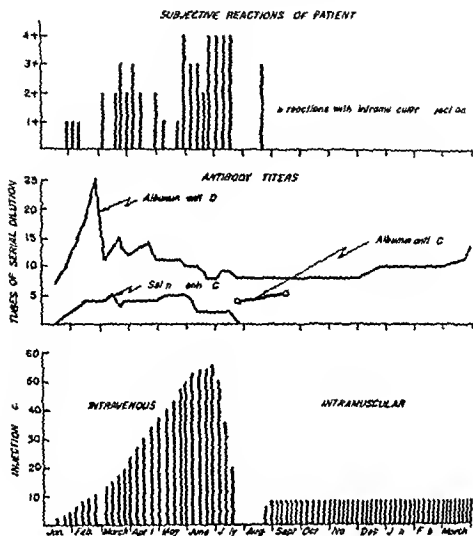


Fig 1—Mrs L. N. E. for details see Table I

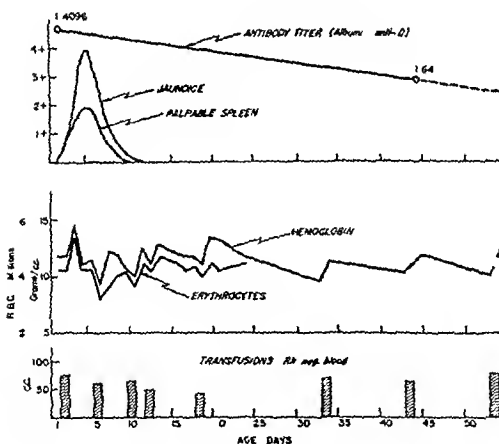


Fig —Baby L. V. E. for details see Table II

The expected date of delivery was April 21, 1948. The course of the pregnancy was uneventful with an average gain in weight of the mother and with fetal size, activity, and heart sounds being normal. Since the antibody titer had shown a continued rise from the twenty-third week of the pregnancy, a surgical induction with rupture of the membranes was performed on March 29,

TABLE II

DATE (1948)	WEIGHT (LB OZ)	HEMO GLOBIN (GM)	RBC (MIL LIONS)	WBC	ALBUMIN ANTI D	JAUNDICE	SPLEEN	TRANS FUSION
Mar 29	5 12	11 7	4 26	15,750	1 4,096	0	0	
Mar 30	5 8	11 7	4 20	27,400		+	+	75 cc
Mar 31	5 10	14 6	5 69			++	++	
Apr 1	5 9	11 1	4 26			++++	++	
Apr 2	5 8	11 5	4 20			+++	+	
Apr 3	5 6	9 3	3 26			++	+	60 cc
Apr 4	5 7	12 1				+	0	
Apr 5	5 8	11 0	4 09	7,450		+	0	
Apr 6	5 6	11 0	4 20			+	0	
Apr 7	5 8	10 0	3 66			+	0	65 cc
Apr 8	5 10	12 5	4 45			0	0	
Apr 9	5 11	11 0	4 20			0	0	60 cc
Apr 10	5 11	12 8	4 70			0	0	
Apr 11	5 11					0	0	
Apr 12	5 12	12 1	4 45			0	0	
Apr 13	5 13	11 7	4 20			0	0	
Apr 14	5 14	11 7	4 31			0	0	
Apr 15	5 15	11 0	3 97			0	0	40 cc
Apr 16	6 0	13 5	4 56			0	0	
Apr 17	6 1	13 5	4 20	8,500		0	0	
Apr 21	6 8	11 7	4 45			0	0	
Apr 30	7 4	9 5				0	0	70 cc
May 1		11 3				0	0	
May 10	7 13	10 2				0	0	65 cc
May 11		11 8			1 64	0	0	
May 21	8 14	9 1				0	0	80 cc
June 9		9 5				0	0	15 cc
June 21	10 6	10 7				0	0	
July 7	12 14	11 5			0	0	0	
Aug 15	14 1	11 0						
Sept 15	14 10	11 7						
Oct 15	16 8	11 3						
Nov 15		11 7						

Data on infant (Baby L E E) showing weight blood counts Rh antibody titer etc. Jaundice graded from + to +++ the latter indicating a deeply jaundiced condition. Spleen only slightly enlarged. At birth occasional erythroblasts were seen in the blood smear none subsequently. The red blood cell fragility was normal.

Platelets 516 600. X-rays of the skull long bones and chest were negative. All transfusions given were of compatible Rh-negative blood.

1948 (thirty-seventh week), with delivery of a lusty baby girl weighing 5 lb 12 oz (2,600 grams). The amniotic fluid was clear. The placenta was normal grossly and microscopically. On immediate thorough physical examination the infant appeared quite normal without evidence of jaundice or edema. The liver and spleen were not palpable. The following day the baby was slightly jaundiced and the tip of the spleen was palpable. The progress of the baby is recorded in Table II and Fig 2.

The Rh tests on the baby were as follows

(1) Cord blood at birth Group O cde cde (C-, D-, E-, c+) Anti human globulin test (Coombs' test) on the baby's cells was negative

	MOTHER'S SERUM WITH BABY'S CELLS	MOTHER'S SERUM WITH PANEL	BABY'S SERUM WITH PANEL
Saline	None	None	None
Albumin	None	Anti D-1 4 096	Anti D-1 4,096''

This test was repeated the following day on the same sample of blood with identical results \*

(2) Two days of age "Group O Behaves like an Rh negative blood but this is so only because of very intense coating with maternal antibodies"†

(3) Three weeks of age 'Group O Rh negative The cells are not now coated with maternal antibodies Antibodies (anti D) still demonstrable at a titer of 1 64"‡

(4) Three and one half months of age "Group O Rh positive Type cDe cde (R<sub>0</sub> r), (C-, D+, E- c+ C-)"†‡

At date of writing (March 1949), the baby has progressed and developed quite normally

#### DISCUSSION

This case is not presented with any idea of recommending the procedure carried out as a method of treatment, even though the outcome of the pregnancy was a happy one It does however, present several interesting features and raises some question as to the validity of the current concepts of the pathogenesis of erythroblastosis

One interesting feature of the case is the negative antihuman globulin test for 'cell coating' (Coombs' test) on the baby's cells at birth Race<sup>4</sup> offers the following possible explanations of this (a) The baby's cells were not adequately washed free from serum (b) The antihuman globulin was not sufficiently active (c) Though sensitized, the baby's cells failed to give a positive Coombs test (d) At the time of birth the baby's cells were not sensitized and only became sensitized later

Another feature was the consistent absence of or weak agglutination in albumin of the anti D factor (negative prozone) in the first two to five tubes of the titrations Levine, Mohn and Witebsky<sup>5</sup> believe that this prozone phenomenon which usually occurs only in highly immunized individuals may be due to a third type of Rh antibody of true blocking character, in addition to saline (complete) and albumin (incomplete) antibodies They believe that this type of blocking Rh antibody prevents agglutination by either of the others Howard and co workers<sup>6</sup> suggest that blocking antibody may act as a protection for the erythrocytes of the fetus The hypothesis could be offered that this explains the successful outcome in this case.

A remarkably high titer of albumin antibody was obtained in this case This was to be expected with the intensive additional immunizations There

\*Tests performed by the Vancouver Red Cross Laboratory

†Philip Levine M D

‡Confirmed by Dr R R Race The Walter Institute, London England and by Dr L K Diamond, The Blood Grouping Laboratory, Boston Mass.

was no correlation of the antibody titers with the reactions of the mother (Table I and Fig 1). The recovery of an infant with such a high content and long persistence in its blood of maternal antibodies is certainly a rarity. Wiener and co-workers<sup>2, 10</sup> report a case of survival of an infant with massive exchange transfusions. In none of their previous cases had an infant survived if the mother had a titer as high as blocking test to 4 units and the albumin plasma conglutination test to 50 units. Statistics of Davidsohn and Stein<sup>4</sup> show that where blocking antibodies were present to 1:10 or less, 41.9 per cent of infants did not survive, and where the titer was above 1:10, 71.4 per cent did not survive. They also state, in regard to a mother with serum agglutinations to 1:20 and blocking antibodies to 1:5 at the eighth week of pregnancy, that the possibility that the antibodies were of the "carry-over" variety was the only hope in the case.

Carter<sup>11</sup> has very recently had considerable success in lowering antibody titers and preventing erythroblastosis by giving the mother prenatal injections intramuscularly of Rh haptens prepared by alcohol and ether extraction from pooled Rh-positive cells. The hapten<sup>12, 13</sup> also has been used with success in the treatment of erythroblastosis in the infant. It would seem to be a possibility in the case here presented that the breakdown and dissolution of the Rh-positive red blood cells at the sites of the intramuscular injections might have released some hapten for absorption.

#### SUMMARY

A report is presented of an isoimmunized Rh-negative woman. Following transfusion of her husband's Rh-positive blood and delivery of a stillborn, hydroptic infant, additional intensive immunization was brought about by intravenous injections of the husband's blood over a period of six months. An extremely high antibody titer was produced early in the course of the injections which fell rapidly and appeared to be stabilized. Pregnancy supervening and subjective reactions preventing any further intravenous injections, the patient was given a series of intramuscular injections of her husband's blood throughout the pregnancy. She was delivered of a mildly erythroblastotic infant which survived without any great difficulty. Tables and illustrations presenting the course of the mother and infant are included. Various interesting features of the case are discussed.

We would like to thank especially the Vancouver Red Cross Laboratory which performed most of our tests, Dr R. R. Race of The Lister Institute, London, England, who confirmed the family genotypes, Dr R. Galpin, Pediatrician, Victoria, British Columbia, who cared for the baby girl, Dr Bruce Chown, Professor of Pediatrics, University of Manitoba Medical School, who assisted in the preparation of this report, and Dr Philip Levine, The Rh Blood Testing Laboratory, Ortho Research Foundation, Raritan, N. J., who performed a number of the tests for us as noted in the text.

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## A RE-EVALUATION OF PAPAVERINE IN THE TREATMENT OF ANGINA PECTORIS

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FOR several years papaverine has been used, both intravenously and orally, in the treatment of the anginal syndrome with conflicting reports as to its value. It is an isoquinoline alkaloid derived from opium which does not produce addiction<sup>1</sup>. Experimentally it has been shown in studies from this laboratory and elsewhere to be a powerful vasodilator<sup>2, 3, 4</sup>. It diminishes or abolishes premature systoles of ventricular origin<sup>5, 6</sup>. Pal<sup>7</sup> and Macht<sup>8</sup> recorded some beneficial effects with intravenous papaverine. Using oral doses of 26 mg ( $\frac{3}{8}$  gr) daily, Evans and Hoyle<sup>13</sup> reported questionable benefit. In 1942 this department administered oral papaverine in doses higher than those previously used with 400 mg (6 gr) daily there was apparent improvement in 75 per cent of the cases studied<sup>5</sup>. Since this report other authors have obtained divergent results. Swanson<sup>9</sup> reported improvement in eleven of twelve patients. Gray and co-workers<sup>10</sup> found no improvement in ten patients using daily doses of 400 mg (6 gr) and 800 mg (12 gr). White<sup>11</sup> and Gold<sup>12</sup> have both stated that the drug is of little or no value. Our clinical experience with the drug in the years since the initial optimistic report has been disappointing. This experience, plus the variable results of other investigators, demanded a critical reevaluation of the usefulness of papaverine in the treatment of the anginal syndrome.

### METHOD

The present study was carried out on patients attending the out patient Cardiac Clinic of the Michael Reese Hospital. Each of these patients had been attending the clinic for at least one year, and all had been thoroughly investigated. The criteria for selection of patients were (1) each individual must unquestionably suffer from angina pectoris, (2) the attacks must occur at least twice weekly and preferably more often, and (3) the patient must be sufficiently intelligent and cooperative for an evaluation to be made. In selecting cases for this study more than one hundred patients with the clinical diagnosis of angina pectoris were interviewed. Of these only twenty seven seemed to fit our criteria as stated. Of these twenty seven patients, fourteen were dropped as the study progressed. Of these, four improved so much during the control and placebo periods that they no longer sustained the required number of weekly attacks. The remaining ten were dropped for various reasons: consistent failure to keep appointments, inability to attend the clinic frequently because of long distance to travel, and doubt as to the accuracy of the diagnosis. The final group consisted of thirteen patients, six women and seven men, ranging in age from 35 to 73 years with an average of 64. The duration of the anginal syndrome was one to thirteen years with an average of four and one half years. Ten patients had arteriosclerotic heart disease, two had arteriosclerotic and hypertensive heart disease, and one had hypertensive heart disease. Three had definite histories of myocardial infarction.

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A complete history and physical examination were done in each case. Laboratory data included a complete blood count, urinalysis, blood urea nitrogen, chest x-ray, fluoroscopy of the heart and lungs, and electrocardiograms taken before, during, and following papaverine therapy. The patients were seen biweekly, occasionally at three week intervals, usually by the same physician in an effort to establish and keep the patient-physician relationship as a constant environmental factor. Each patient was instructed to keep a diary on forms which were supplied showing the number and duration of attacks and the number of nitroglycerine tablets used each day. On each clinic visit the patient was questioned regarding subjective improvement, degree of activity indulged in during attacks, average number of blocks he could walk before anginal pain occurred, and changes in living habits and the environmental and emotional situation which might be contributory. All received nitroglycerine as required. In some instances other medication such as phenobarbital was used. The amounts of other medication were maintained constant. Three patients were on a maintenance dose of digitalis throughout the study.

At the beginning of the study each patient was followed for at least six weeks before either placebo or papaverine medication was administered. This period served as one of adjustment for the patient and physician. When it appeared that the patient's angina had reached a basic level, he was given placebo (lactose) capsules which appeared in all respects identical with the papaverine capsules\*. These were taken four times daily before meals and at bedtime and the patient was told that the capsules were to reduce or prevent chest pain. After not less than four weeks on placebo therapy, during which time any improvement was evaluated, capsules containing 100 mg ( $1\frac{1}{2}$  gr) of papaverine hydrochloride were substituted for the placebo without the patient's knowledge. This dosage level was maintained for four weeks or more. In order to minimize the effects of spontaneous variations in the course of the disease and the psychic influence of drug administration, the patient was kept at various dosage levels of placebo or papaverine until his response appeared to have stabilized. When a definite decision could be made regarding response to the 400 mg (6 gr) daily dosage, the drug was increased to 600 mg (9 gr) a day, later to 800 mg (12 gr) daily. If there was any doubt about the response to papaverine, placebo capsules were substituted at the same dosage level. Finally, following cessation of placebo and papaverine therapy, each patient was followed for a further control period of at least four weeks. The total period of observation covered eight to twelve months.

#### CRITERIA FOR EVALUATION

The evaluation of therapy for chronic coronary insufficiency when manifested by the anginal syndrome is always a difficult task. There are at least three major reasons for this: (1) spontaneous variations in the course of the disease; (2) the nonecontrollable psychic influences; (3) the absence of a reliable quantitative test to judge the effect of treatment. We believe that these factors have been mainly responsible for the variable results obtained by the different investigators who have attempted to evaluate the efficacy of papaverine in angina pectoris. The present study was undertaken with a view toward obtaining experimental conditions which would minimize the importance of the foregoing factors as much as possible. Directed toward this end were the careful selection of cooperative patients, the observation of the patient through a relatively long initial control period, and maintenance of the patient on placebo and successive dosage levels of papaverine until the response was clear.

In planning the study, consideration was given to the use of the exercise tolerance or anoxemia tests as possible objective (hence more reliable) methods to judge the effect of treatment. These tests were not used for the following reasons:

\*We are grateful to Eli Lilly & Company for supplying the papaverine and placebo capsules used in this study.

1 It would be impossible to assess the validity of changes in the patient's response to exercise or anoxemia without establishing a long control period during which these tests would have to be repeated as frequently as one or more times per week and as frequently during the placebo and drug periods. Such a program was impracticable. Furthermore, there is no evidence in the literature to indicate that either of these tests has or can be used as a fine reliable quantitative measure of the degree of coronary insufficiency.

2 Both the exercise tolerance and anoxemia tests are artificial procedures. Neither one can be considered an accurate reproduction of the total clinical state which results in angina pectoris. It was felt, therefore, that results from these tests could not be applied directly to the anginal syndrome as seen clinically. The efficacy of drug therapy in the treatment of angina pectoris can be determined only by observations on the naturally occurring syndrome.

Judging the effect of treatment in angina remains a difficult problem in clinical evaluation. When observed over periods of many months, patients with angina display wide variations in ability to walk distances and perform routine tasks, and striking differences in the number of attacks of pain suffered. Basing indications of improvement on either severity or duration of attacks or the number of nitroglycerine pills used in a given period might be misleading, although considered together these data suggest a trend. We considered the most useful criterion of the patient's course to be the number of attacks of pain suffered in a given period. It is true that with improvement the patient may increase his activity and thereby keep the attacks of pain at a constant frequency, however, the opposite is also true. Therefore, if the drug being investigated produces a striking or dramatic decrease in the patient's attacks of chest pain, there will be no doubt in the mind of the investigator as to the efficacy of his measures. Marginal results are difficult to evaluate. A dramatic result was considered to be an abrupt decrease by at least 50 per cent in the attacks of chest pain when they numbered in excess of forty per week, 75 per cent when they were between ten and forty, and 100 per cent when they were ten or less.

#### RESULTS

The results are summarized in Table I. All thirteen patients received a trial on 400 mg (6 gr) daily of papaverine. In only two patients was there a significant reduction in the number of attacks of pain. One of these (J S) showed a further reduction when the dosage was increased to 600 mg (9 gr) daily. However, at the higher level he complained of severe weakness and anorexia, and the papaverine had to be discontinued. Unfortunately the patient died suddenly before the final control period could be evaluated. The other patient (S M) who showed improvement on 400 mg (6 gr) daily was unusually sedated by the drug. Instead of his customary complaints of insomnia the patient now slept twelve to fourteen hours each night so that he no longer had frequent anginal attacks at night. This patient was so severely constipated by the papaverine that the drug had to be stopped.

Nine patients received 600 mg (9 gr) daily. Only the one already discussed (J S) showed significant decrease in the frequency of pain. Seven pa-

TABLE I  
AVERAGE WEEKLY NUMBER OF ATTACKS OF PAIN  
PAPAVRINE 1ET DAY

PATIENT	AGE	SEX	TYPE OF DISEASE	PAPAVRINE 1ET DAY												CONTROL	PLACEBO	CONTROL
				CONTROL	PLACEBO	6 GR	9 GR	12 GR	12 GR	12 GR	12 GR	12 GR	12 GR	12 GR	12 GR			
ES	53	F	Arteriosclerotic	8 (6)	9 (5)	7 (6)	8 (2)	8 (2)	7 (4)	7 (4)	7 (4)	7 (4)	7 (4)	7 (4)	7 (4)	7 (18)	95 (12)	0 (5)
WM	63	M	Arteriosclerotic	10 (11)	36 (8)	10 (4)	6 (-)	63 (6)	93 (2)	93 (2)	93 (2)	93 (2)	93 (2)	93 (2)	93 (2)	95 (12)	95 (12)	0 (5)
AT	57	F	Arteriosclerotic and hypertensive	4 (13)	2 (14)	2 (5)	-	-	0.1 (7)	0.1 (7)	0.1 (7)	0.1 (7)	0.1 (7)	0.1 (7)	0.1 (7)	0 (5)	0 (5)	0 (5)
AL	3	M	Arteriosclerotic	14 (6)	7 (10)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)	8 (12)
AM	57	M	Arteriosclerotic	15 (14)	15 (12)	16 (12)	23 (4)	-	21 (8)	21 (8)	21 (8)	21 (8)	21 (8)	21 (8)	21 (8)	21 (8)	21 (8)	21 (8)
MM	68	F	Arteriosclerotic	10 (6)	15 (5)	14 (3)	12 (7)	-	14 (3)	14 (3)	14 (3)	14 (3)	14 (3)	14 (3)	14 (3)	14 (3)	14 (3)	14 (3)
LI	69	M	Arteriosclerotic	7 (8)	83 (4)	95 (4)	91 (2)	81 (2)	84 (2)	84 (2)	84 (2)	84 (2)	84 (2)	84 (2)	84 (2)	84 (2)	84 (2)	84 (2)
LS	67	M	Arteriosclerotic	9 (6)	64 (4)	40 (4)	25 (2)	-	0 (7)	0 (7)	0 (7)	0 (7)	0 (7)	0 (7)	0 (7)	0 (7)	0 (7)	0 (7)
OS	33	F	Hypertensive	12 (10)	0.1 (19)	0.1 (10)	13 (3)	10 (2)	53 (4)	53 (4)	53 (4)	53 (4)	53 (4)	53 (4)	53 (4)	53 (4)	53 (4)	53 (4)
RB	68	F	Arteriosclerotic and hypertensive	10 (9)	8 (4)	8 (4)	6 (2)	6 (2)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)
AM	50	F	Arteriosclerotic	9 (6)	6 (6)	5 (6)	6 (2)	6 (2)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)
ES	50	M	Arteriosclerotic	17 (6)	14 (5)	14 (6)	15 (2)	18 (2)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)	14 (5)
SM	52	M	Arteriosclerotic	35 (12)	42 (5)	10 (20)	10 (11)	18 (2)	40 (10)	40 (10)	40 (10)	40 (10)	40 (10)	40 (10)	40 (10)	40 (10)	40 (10)	40 (10)

The numbers in parentheses indicate the duration of each observation period expressed in weeks

†This patient died suddenly shortly after papaverine was discontinued

‡This patient lost 34 pounds in weight during the period of observation. The decrease in anginal attacks and improvement in ability to work paralleled weight loss

While taking 12 gr of papaverine daily this patient's anginal attacks became very frequent. Papaverine was decreased to 6 gr daily for a four week period without change in symptom. This was followed by a trial of placebo without significant change. During the final control period there was a gradual lessening of attacks. There was no electrocardiographic evidence of myocardial infarction. This patient lost 57 pounds in weight during the period of observation and 61 pounds in a period of one year without affecting anginal syndrome

tients received 800 mg (12 gr) daily. In no case was there a clear cut improvement in the ability to perform routine activities at any dosage level of papaverine.

Toxic symptoms were observed at the 400 mg (6 gr) level in two patients. In both the complaints were of drowsiness and constipation. With the dosage of 600 mg (9 gr) daily one patient complained of weakness and anorexia and one of headache and epigastric distress. At the 800 mg (12 gr) daily level one patient complained of drowsiness and two of constipation.

#### DISCUSSION

This series is large enough and sufficiently well controlled to warrant the deduction that large doses of papaverine do not have a uniformly beneficial effect on angina pectoris distinct from a psychic action. It is possible that the random sample selected is weighted and the negative results obtained are not typical of a larger series, but even if this were so, the present study demonstrates that papaverine administered orally is of limited value in angina pectoris. This does not preclude its trial clinically since an occasional patient may be found in whom the drug has a striking, beneficial effect without untoward side reactions.

The present study differs from our earlier study in one very important respect, that is the criterion of improvement. If this criterion of a striking or dramatic reduction in the number of attacks of chest pain were applied to the earlier study it would be found that many cases classified as definitely improved would now be considered as marginal results. Thus, using our present more critical attitude the results of the two studies would not be far apart.

Since papaverine definitely acts as a powerful coronary dilator in animals, its failure to produce clinical results in man may be on the basis of one or several mechanisms. (1) Its action on the coronary vessels of man, particularly sclerotic coronaries, may be different from its action in animals. (2) Coronary dilatation in the cardiac region responsible for the anginal pain may not be great enough, or may be neutralized by dilatation of other systemic vessels with which the coronary vessels form parallel circuits. (3) Factors other than inadequate coronary flow may dominate the mechanism giving rise to anginal pain. (4) Dramatic results in patients with anginal pain, aside from psychic effects, may require a quick acting as well as a powerful coronary dilator such as nitroglycerine. Slower acting dilator drugs may permit readjustments not obtainable when a quick-acting drug is used.

#### CONCLUSIONS

1. The oral administration of papaverine in doses of 400 mg (6 gr) to 800 mg (12 gr) daily did not significantly reduce the frequency of attacks of angina pectoris or increase the patients' ability to perform routine activities. In eleven of thirteen cases followed over a period of eight to twelve months.

2. In two patients papaverine appeared to have a beneficial effect on the anginal syndrome. In one patient the results appeared to be due to the sedative effect of the drug, in the other the cause of improvement was not certain.

3 Toxic effects were noted in two patients taking 400 mg (6 gr) daily, two taking 600 mg (9 gr) daily, and three taking 800 mg (12 gr) daily. Constipation and drowsiness were the most common symptoms.

4 A careful reevaluation of the use of oral papaverine in the treatment of the anginal syndrome leads us to conclude that it is of limited value.

We are grateful to the clinic physicians for permission to use their patients in this study.

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# SEMIWEEKLY TREATMENT OF SYPHILIS WITH PROCAINE PENICILLIN IN OIL

REACTIONS AND PRELIMINARY RESULTS IN 228 PATIENTS

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THE superiority of penicillin over previous antisyphilitic agents has been clearly demonstrated by the results of the nationwide study of penicillin in syphilis<sup>1</sup> Early penicillin preparations, administered in aqueous solution, required hospitalization of the patient for frequent injections With the development of penicillin in peanut oil and wax by Romansky and Rittman,<sup>2</sup> the ambulatory treatment of syphilis was made possible, and results thus far available indicate that schedules employing daily injections of this material are at least as effective as aqueous regimens of similar duration More recently, longer acting repository penicillin preparations have been produced It is the purpose of this report to describe the use of one of these, crystalline G procaine penicillin in sesame oil, administered at more prolonged intervals (semiweekly) in the ambulant treatment of syphilis

The objectives of the present study were twofold (1) to determine the therapeutic efficacy of procaine penicillin in oil administered semiweekly in the treatment of syphilis, and (2) to determine the practicability of a semiweekly schedule in the treatment of ambulant patients from the standpoint of ease holding The preliminary results herein presented are based on the treatment of 228 patients with all stages of syphilis At the same time, information has been obtained on the lack of toxicity of procaine penicillin in oil, particularly in respect to the procaine fraction and its continued administration over prolonged periods

## MATERIALS AND METHODS

Procaine penicillin, a slightly soluble salt, is prepared by the chemical combination of procaine hydrochloride and sodium penicillin G<sup>3</sup> The preparation employed (Duracillin, in oil\*) is suspended in sesame oil, with dispersing agents added, and contains 300,000 units of penicillin and approximately 125 mg of procaine per milliliter Ninety per cent of the penicillin is penicillin G with a potency of 940 units per milligram, and at least 50 per cent of the particles are of large size, 50  $\mu$  or more in diameter<sup>3</sup> Because of low solubility, large particle size, and suspension in oil, the penicillin is slowly absorbed and produces prolonged serum concentrations Following a single intramuscular injection of 600,000 units, the dosage employed in this study, detectable serum penicillin concentrations have been observed at forty eight hours in as many as 89 per cent of patients,<sup>4</sup> and excretion of penicillin in the urine continues for longer periods of time Because of its slow absorption, procaine penicillin in oil would appear on a priori grounds to be well suited for the treatment of syphilis

In a like manner the procaine is released slowly from its combination with penicillin and is absorbed over a period of hours Although widely used for its local anesthetic properties, and recently administered intravenously in the treatment of arthritis and other disorders,<sup>5</sup> occasional instances of intolerance or sensitivity to procaine have been reported<sup>6</sup>

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In the present study, because of concern in respect to the rather large amount of procaine contained in the preparation (approximately 250 mg per 2 ml dose) an attempt was made initially to screen patients in order to detect those who c tolerance to procaine might be low. Consequently each patient was questioned regarding previous procaine injections, e.g., for extraction of teeth, skin tests were performed by the injection of 0.1 ml of 2 per cent aqueous procaine solution (2 mg) intradermally and, in reference to systemic intolerance 0.5 ml (10 mg) was injected intramuscularly. After thirty minutes of observation, an initial dose of 300,000 units of procaine penicillin in oil was injected; all subsequent injections being of 600,000 units each. On each of the first three return visits, patients were questioned by a physician regarding both local discomfort and general reactions and the injection site was inspected and palpated.

**Regimens Employed**—The results of the national study of penicillin in syphilis have shown essentially similar failure (or retreatment) rates for aqueous penicillin with schedules varying in duration from four to fifteen days in dosage from 12 to 48 million units,\* and in interval between injections from two to six hours.<sup>9, 10</sup> Results with penicillin in peanut oil and wax administered either daily or twice daily for eight and for fifteen days in total dosages of 48 to 96 million units have not been significantly different.<sup>11, 12</sup> Because prolongation rather than intensification may prove to be the important factor in the treatment of syphilis and because of previous experience on a small scale using penicillin in peanut oil and wax schedules involving semiweekly injections of 600,000 units of procaine penicillin in oil were chosen. Experimental support for increasing the total duration of treatment has been presented by Eagle, Magnuson and Fleischman<sup>13</sup> who obtained successful results in treating rabbit syphilis with a schedule consisting of intramuscular injections of only 7 units of aqueous penicillin per kilogram of body weight over a prolonged period. It is significant that with this regimen Eagle and co-workers were at no time able to demonstrate detectable serum penicillin concentrations. Low concentrations acting over a long period of time were more effective than high concentrations acting over a short period of time. Subsequently, the same authors<sup>14</sup> found penicillin in peanut oil and wax to be two to fourteen times more effective than aqueous solutions in the treatment of experimental syphilis. For these reasons, therefore the duration of treatment of patients included in the study was arbitrarily prolonged to seven and one-half weeks (total dosage 90 million units) or alternately, in some patients with more serious late forms of the disease to ten weeks (120 million units).

**Clinical Material**—All new patients with syphilis regardless of stage of disease were treated with one or the other of the foregoing schedules. The diagnosis and treatment regimen in 28 patients are indicated in Table I. The division between early and late latent syphilis is based on the two year time period.

TABLE I. DIAGNOSIS AND TREATMENT REGIMEN EMPLOYED IN 28 PATIENTS

SCHEDULE	TOTAL DOSEAGE	90 MILLION UNITS	120 MILLION UNITS	TOTAL
	DURATION	7½ WK	10 WK	
DIAGNOSIS		NUMBER OF PATIENTS	NUMBER OF PATIENTS	
Early (primary or secondary)		24		24
Early latent		22		22
Late latent		39		39
Neurosyphilis				
Early asymptomatic		1	2	3
Late asymptomatic		17	20	37
Late meningovascular		3	10	13
Paretic		1	-	1
Tabetic		8	10	18
Cardiovascular		9	3	12
Benign late (gummatous)		6		6
Congenital (interstitial keratitis)			1	1
Total		150	48	198

\*and to 96 million units in our own material

## RESULTS

*Toxicity —*

*Procaine* Of forty-four patients subjected to the screening procedure for procaine toxicity, none reported previous untoward reactions to the drug. The intradermal test was interpreted as negative in forty-two and doubtful (slight erythema) in two. Although the intramuscular test dose resulted in no evidence of procaine intolerance, one patient reported suggestive symptoms following the injection of procaine penicillin in oil. This patient, a 38-year old white woman with late neurosyphilis recently receiving treatment with the arsenicals and bismuth, was unaware of any previous procaine injection. She was one of the two patients whose skin test showed erythema and, consequently, although 10 mg of procaine alone intramuscularly resulted in no subjective evidence of intolerance, she received only 150,000 units of procaine penicillin in oil as the initial dose. There were no immediate untoward symptoms but about ten hours later she awoke with feelings of extreme apprehension, nervousness, dyspnea, tightness in the chest, palpitation, tachycardia, and generalized numbness. These symptoms, varying in intensity but gradually diminishing, persisted for three days. Further injections of procaine penicillin in oil were refused, and treatment was subsequently carried out without difficulty with penicillin in peanut oil and wax. The symptoms described are compatible with procaine intolerance, but proof of its causal relationship is lacking.

Although the pretreatment procaine survey was discontinued after the original series of forty-four patients had been completed, all patients receiving procaine penicillin in oil were questioned on each return visit by the treatment nurse and at frequent intervals by a physician, and no further instances of suspected procaine intolerance occurred.

*Penicillin* Of the 212 patients who have completed treatment, two with pre-existing dermatophytosis pedis developed, after the first injection of procaine penicillin in oil, transitory vesiculopapular eruptions limited to the feet which disappeared promptly without interruption of treatment. Five patients developed generalized pruritic eruptions (papular in two, urticarial in three) during the course of treatment. In one patient, who one year previously had developed a giant hive at the site of injection of aqueous penicillin for pneumonia, generalized urticaria appeared after the first injection of procaine penicillin in oil. In two patients the generalized eruptions appeared after the fourth injection, and in one each after the sixth and seventh injections. In two of these five patients treatment was interrupted temporarily, in three, itching was controlled with an antihistaminic drug and treatment was continued uneventfully.

These eruptions were similar to those observed previously in patients treated either with aqueous penicillin or with penicillin in peanut oil and wax. The incidence of this type of reaction following procaine penicillin in oil (approximately 2.5 per cent of patients) is less in our experience than with penicillin in peanut oil and wax (5 per cent).



The procaine penicillin in oil preparation has proved superior to penicillin in peanut oil and wax in other respects. These include ease of handling and of administration, and absence of local discomfort and of nodule formation such as was observed following the inadvertent superficial injection of penicillin in oil and wax. Rarely, and because of faulty technique of injection, transient numbness of the leg on the injected side has occurred.

In summary, in approximately 3500 injections administered to a total of 228 patients, of whom 212\* have received from fifteen to twenty injections over seven and one half to ten week time periods, there has been only a single instance of suspected (but not proved) procaine intolerance and an incidence of 25 per cent or less of generalized eruptions of the type attributed to penicillin. From the standpoint of ease of administration and absence of local discomfort, this procaine penicillin in oil preparation has proved highly satisfactory.

#### THERAPEUTIC EFFICACY

In a chronic infection such as syphilis little information on the therapeutic effectiveness of an agent can be gained from a small series of patients with diverse stages of disease observed for only brief periods of time. It is to be emphasized that the data to be presented are meager, and final interpretation must wait upon future experience. Preliminary impressions of therapeutic effectiveness can be obtained (1) in early syphilis by the disappearance time of *Treponema pallidum*, by the rapidity of healing of lesions, by the serologic response and by the occurrence of relapse (2) by the healing of lesions in benign late syphilis and (3) by the trend of the cerebrospinal fluid abnormalities in neurosyphilis.

*Early Syphilis*—*T. pallidum* disappears rapidly (within twenty four hours) from the moist lesions of early syphilis following injection of an effective antitreponemal agent. In the twenty four patients with early syphilis treated with the semiweekly schedule (600,000 units per dose), dark field examination was frequently impracticable at the time of the first post treatment visit because of the advanced stage of healing. For this reason ten patients with dark field positive lesions of early syphilis† were observed eighteen to twenty four hours after an intral injection of 300,000 units of procaine penicillin in oil. In each instance *T. pallidum* were no longer present in serum expressed from previously dark field positive lesions‡. This corresponds with previous findings following penicillin either aqueous or in oil and wax.

As indicated the lesions of early syphilis promptly resolved this is in conformance with observations following the administration of other penicillin preparations.

In respect to serologic response the data are too meager for statistical presentation. However, as of this writing, eight of twenty four patients have

\*Sixteen patients lapsed before completing treatment.

†Not otherwise included in this report.

‡The author wishes to thank Dr Leland Hanchett Medical Director Midwestern Medical Center for these observations.

achieved seronegativity, the remainder have doubtful or low-titered quantitative tests. None have relapsed either clinically or serologically, there have been no reinfections.

It should be emphasized that none of the above-mentioned criteria, other than relapse, are reliable indicators of therapeutic efficacy. The adequacy of any therapeutic agent (in early syphilis) can be determined only by the careful and prolonged observation of large numbers of patients for evidence of clinical and serologic relapse. However, there is reason to believe that procaine penicillin in oil will prove to be at least as effective as penicillin in oil and wax.

The lesions of benign late syphilis heal after even relatively minute amounts of aqueous penicillin.<sup>17</sup> The prompt response of the six patients with gummas (two cutaneous, one mucosal, two visceral) treated with procaine penicillin in oil would be anticipated.

*Neurosyphilis*—It has been emphasized by Dattner<sup>18</sup> that the most reliable indicator of therapeutic effectiveness in neurosyphilis is the response of the cerebrospinal fluid abnormalities, particularly in terms of cell count and total protein. Improvement in colloidal and quantitative complement fixation tests follows slowly and may require several years to become manifest. Of the seventy-four patients with neurosyphilis included in the present study, twenty-five have thus far had examinations of the cerebrospinal fluid approximately three to four months following completion of treatment. Evidence of "activity" of the syphilitic process before treatment is documented by the following data. Using the method devised by Reynolds,<sup>19</sup> but employing median values rather than means as a more representative centering constant for small series, the results of the pretreatment cerebrospinal fluid examination in these twenty-five patients were as follows: cell count =  $92/3$ ,\* total protein = 64 mg per cent, colloidal gold = 15,† complement fixation test =  $16 \pm$ . The initial post-treatment results in these same patients obtained three to four months following completion of treatment are in striking contrast: cells =  $10/3$ , total protein = 46 mg per cent, colloidal gold = 3, complement fixation test = 4.

The three following brief case reports are typical of the cerebrospinal fluid response in patients observed for more prolonged periods of time. Although none thus far have required re-treatment, much longer observation will of course be necessary to determine the ultimate outcome.

CASE 1—A. B. (A 4271) a 41 year old white man with untreated late meningovascular neurosyphilis, received 12 million units of procaine penicillin in oil (600,000 units twice weekly) between January 30 and April 6, 1948. The cerebrospinal fluid results are indicated in Table II. The blood serologic titer over the same period decreased from 600 to 120 Kohn units.

\*Fuchs-Rosenthal counting chamber: 92 cells per  $3.2 \text{ mm}^3$  of spinal fluid.

†Arithmetic sum of the first three tubes: e.g.  $5554321000 = 15$ .

‡In this laboratory the spinal fluid complement fixation test is quantitated by using decreasing amounts of spinal fluid as follows: 1.0, 0.5, 0.25, 0.12, 0.06, 0.03, 0.01, and the result of each reported. In accordance with Reynolds' reciprocal values were assigned to the various readings: 0.01=100, 0.03=33, 0.06=16, 0.12=8, 0.25=4, 0.5=2, 1.0=1.

TABLE II CEREBROSPINAL FLUID RESPONSE IN CASE 1

DAYS BEFORE OR AFTER END OF TREATMENT	CELLS/MM <sup>3</sup>	TOTAL PROTEIN (MG %)	COLLOIDAL GOLD	COMPLEMENT FIXATION TEST									
				AMOUNT OF SPINAL FLUID (ML)									
				0.01	0.03	0.06	0.12	0.25	1.0	2.0	3.0	4.0	5.0
-66	180/3	114	55543-100	0	0	±	4	4	4	4	4	4	4
+98	16/3	116	2-11100000	0	0	±	3	3	3	3	3	3	3
+192	25/3	No data	0000000000	0	0	±	1	3	3	3	3	3	3
+290	7/3	91	1111000000	0	0	±	7	4	4	4	4	4	4

\*In this and in subsequent table the number refers to days before the end of treatment, to immediately preceding the beginning of treatment

CASE 2—J M (A 7443) a 49 year old colored man with a entirely untreated (two bi-month injections even years previous) late asymptomatic neurosyphilis of twenty years duration received fifteen injections of procaine penicillin in oil, total dosage 9 million units between Dec 19 1947 and Feb 6 1948. The cerebrospinal fluid response is recorded in Table III. The blood serologic titer remained unchanged during this interval.

TABLE III CEREBROSPINAL FLUID RESPONSE IN CASE 2

DAYS BEFORE OR AFTER END OF TREATMENT	CELLS/MM <sup>3</sup>	TOTAL PROTEIN (MG %)	COLLOIDAL GOLD	COMPLEMENT FIXATION TEST									
				AMOUNT OF SPINAL FLUID (ML)									
				0.01	0.03	0.06	0.12	0.25	1.0	2.0	3.0	4.0	5.0
-107	947/3	163	555433-000	0	3	3	3	3	4	4	4	4	4
+71	38/3	62	5553210000	No data									
+253	5/3	48	1111000000	0	0	?	4	4	4	4	4	4	4

\*See footnote to Table II

CASE 3—P M (A 7444) was a 7 year old colored man with late asymptomatic neurosyphilis of twenty one years duration. Previous treatment administered elsewhere included fever therapy 1 injection of Mapharsen twelve of bismuth and nineteen of Aldarson between March and December of 1946. As recorded the cerebrospinal fluid on admission here one year later showed evidence of marked activity. Between Dec 19 1947 and March 2 1948 he received 12 mill on units of procaine penicillin in oil (600 000 units twice weekly). The cerebrospinal fluid results are recorded in Table IV. The blood serologic titer, low at the onset remained unchanged during this observation period.

TABLE IV CEREBROSPINAL FLUID RESPONSE IN CASE 3

DAYS BEFORE OR AFTER END OF TREATMENT	CELLS/MM <sup>3</sup>	TOTAL PROTEIN (MG %)	COLLOIDAL GOLD	COMPLEMENT FIXATION TEST									
				AMOUNT OF SPINAL FLUID (ML)									
				0.01	0.03	0.06	0.12	0.25	1.0	2.0	3.0	4.0	5.0
-9	43/3	91	5554321000	0	4	4	4	4	4	4	4	4	4
+108	8/3	27	0000000000	0	0	0	2	4	4	4	4	4	4
+276	1/3	31	1111100000	0	0	0	4	4	4	4	4	4	4

See footnote to Table II

The results obtained thus far in patients with neurosyphilis are comparable to those previously obtained by us using aqueous penicillin in similar amounts administered in divided doses every three to four hours to hospitalized patients over two to three week time periods. The advantage of the present method is its applicability to ambulant patients.

It should be emphasized that the particular schedules employed in this study are empirical and that no special virtue can be claimed for semiweekly injections over the two time periods arbitrarily chosen. It seems probable that a variety of regimens e.g. daily three weekly etc. over varying periods of time will prove equally effective.

## HERXHEIMER REACTIONS

An evaluation of the Jarisch-Herxheimer phenomenon requires hospitalization of the patient, particularly in respect to the occurrence of fever. Since one of the objectives of the present study was to determine the practicability of procaine penicillin in oil in the treatment of ambulatory patients, only six patients were in the hospital at the time of their initial treatment. Three of these developed febrile reactions (tabetic neurosyphilis, 38.1° C, parietic neurosyphilis, 39° C, gumma of the liver, 40° C), three did not (primary syphilis, early latent syphilis, syphilitic aortic regurgitation). The time interval between the injection of procaine penicillin in oil and the height of the fever (six to sixteen hours) was the same as with aqueous penicillin. It can be said, therefore, that Herxheimer reactions of the febrile type occur with this penicillin preparation but data as to the incidence are not available in our material.

In view of current concern over the possible occurrence of the Jarisch-Herxheimer phenomenon in cardiovascular syphilis,<sup>15, 16</sup> it is of interest that no clinical evidence of such a reaction was detected in any of twelve patients with either syphilitic aortic regurgitation or aneurysm, or both. Nine of these had received prior treatment (recent or remote) with the metal antisypilitic agents, three were untreated previously. No special precautions were employed in this group of patients. In the seventy-four patients with neurosyphilis, of whom the majority had active disease as reflected by the cerebrospinal fluid abnormalities, headache after the initial injection was rare and neither severe nor prolonged.

From the standpoint of Herxheimer reactions it would appear that the majority of patients with late syphilis can be treated safely on an ambulant basis. This statement is predicated on the assumption that a careful and complete medical history and physical examination do not reveal evidence of such lesions as gumma of the larynx or of the brain where Herxheimer reactions of a serious nature might occur. In patients with parietic neurosyphilis, because of the very nature of the disease as well as because of the high incidence of Herxheimer reactions, hospital rather than ambulant treatment is to be preferred. Although no convincing evidence of the occurrence of this type of reaction has been presented in cardiovascular syphilis, the advisability of routinely instituting treatment with penicillin remains to be determined.

## CASE HOLDING

Of primary importance from the public health standpoint is the number of patients who fail to complete treatment administered on an ambulatory basis. Therefore, in the evaluation of schedules for the outpatient treatment of syphilis, the success or failure of case holding is a paramount concern. Dexter,<sup>20</sup> in reporting the results of semi-intensive arsenotherapy (three injections of Mapharsen and one of bismuth per week for ten to fifteen weeks) at the Johns Hopkins Syphilis Clinic, recorded a lapse rate of 40 per cent. Others (cited by Dexter) using similar schedules have had equally poor success in

case holding. With penicillin in peanut oil and wax, on the other hand, lapses appear to have been distinctly less. For example, Hayman,<sup>21</sup> with schedules consisting of daily injections for six to ten days reported that 87 per cent of 110 patients completed treatment without interruption. Similarly in Chope and Malcolm's<sup>22</sup> series 96.6 per cent of 437 patients completed a ten day penicillin schedule, and Frost<sup>3</sup> reported that 98 per cent of 952 patients completed ambulatory treatment with penicillin Mapharsen, and bismuth on two schedules one of ten and one of twenty four days' duration. It is clearly apparent that in general the shorter the schedule the fewer the patients who will fail to complete treatment. A second factor is the untoward reactions (or lack of them) resulting from the antisypilitic agent employed. Other factors which need not be considered here include patient education, case holding personnel, case holding methods employed, etc.

Schedules used in the present study are longer in duration than those employing penicillin cited (seven and one half and ten weeks as compared with six to twenty four days) and the percentage of lapsed patients is in general slightly greater. Of the 228 patients sixteen (7 per cent) are considered to have lapsed. Six of these lapsed only after they had received more than 60 million units of penicillin and probably had adequate treatment. Other things being equal it would appear that ambulatory regimens employing penicillin will prove superior to arsenical bismuth schedules from the standpoint of case holding.

#### SUMMARY AND CONCLUSIONS

1. Procaine penicillin in oil was administered semiweekly to 228 patients in the ambulatory treatment of syphilis.

2. Two arbitrary schedules of therapy have been used: 600,000 units being injected intramuscularly semiweekly for seven and one half weeks or alternatively for ten weeks.

3. In respect to toxicity one instance of suspected (but not proved) procaine intolerance was observed. No evidence of chronic procaine toxicity developed.

4. Cutaneous reactions of the type attributed to penicillin occurred in five patients (less than 2.5 per cent).

5. On the basis of preliminary evidence this type of repository penicillin seems well suited for the ambulant treatment of syphilis. The criteria considered included disappearance time of *T. pallidum*, rapidity of healing of lesions, and serologic trend in early syphilis, and the response of the abnormalities of the cerebrospinal fluid in late neurosyphilis. Final evaluation of the schedules employed in the present study must await wider application and more prolonged observation.

6. No serious reactions of the Jarisch Herxheimer type occurred.

7. From the standpoint of case holding 93 per cent of 228 patients completed treatment, 7 per cent lapsed. In this respect the ambulant treatment of syphilis with penicillin appears to be more successful than with arsenical bismuth regimens of comparable duration.

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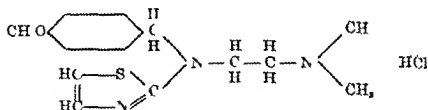
## HISTAMINE ANTAGONISTS

### XIV AN EXPERIMENTAL AND CLINICAL STUDY OF N,N-DIMETHYL-N 2 THIAZOLYL N'p METHOXYBENZYL ETHYLENEDIAMINE HYDROCHLORIDE (194 B)

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ALTHOUGH the antihistaminic drugs currently available are valuable agents for producing symptomatic relief in the management of allergic diseases the results attained have not measured up to the earlier enthusiastic clinical claims for these substances. Their deficiencies include failure to produce relief of sufficient degree and duration, failure to benefit certain types of allergic manifestations, failure to relieve severe allergic symptoms, and the occurrence of certain toxic side reactions. For these reasons new compounds of the ethylenediamine series continue to be synthesized for experimental assay in laboratory animals and clinical trial in human allergy.

We have been studying several of these new substances during the past year. One of them is N,N-dimethyl-N 2 thiazolyl N'p methoxybenzyl ethylenediamine hydrochloride (194 B)\*. It was found to be a good drug for clinical use combining effectiveness with a low incidence of undesirable toxic symptoms.



Laboratory studies by Kitzes, Sondern, and Neary† have shown that compound 194-B has potent antihistaminic properties and low acute and chronic toxicity. We employed several procedures to determine the antihistaminic potency of this drug in guinea pigs. Intraperitoneal injections of 0.1 mg. per kilogram protected all animals against 100 of histamine (0.4 mg. base) given intravenously. The inhalation of a 2 per cent aerosol of this compound for five minutes at 5 pounds pressure released through a DeVilliss nebulizer permitted the animals to remain ten minutes or longer in an aerosol of histamine obtained from a solution of 0.5 mg. per cubic centimeter without showing dyspnea. The same animals when unprotected would have marked dyspnea in two minutes or less. The duration of protection conferred by the aerosol was about seventy five minutes. The intraperitoneal injection of 5

\* From the Department of Internal Medicine, Division of Allergy and the Allergy Research Laboratory, Northwestern University Medical School.

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† Kitzes, C., Sondern, C. W., and Neary, E. R., New Antihistaminic Compounds. N. Arkady Derivatives of N 2 Thiazolyl N,N-Dimethylethylamine. I. Pharmacologic Study. (In press.)

mg of this compound per kilogram was required to give protection against the same histamine aerosol. The duration of this action was about two and one-half hours.

In a group of ten guinea pigs which were actively sensitized with horse serum, 10 mg per kilogram of this compound intraperitoneally were sufficient to prevent anaphylactic death from an intracardial shocking dose. In a control series of unprotected animals the mortality was 85 per cent.

We previously had devised a method to assay or quantitatively titrate the ability of an antihistaminic drug to inhibit the local reaction produced by histamine on the human skin. Essentially the method consists of applying dilutions of the drug to a series of scratches on the skin, and afterwards testing these sites with a dilution of histamine which had previously been determined to give half maximal reactions. Several antihistaminic agents can be assayed simultaneously on one subject, and twenty to forty subjects are used for each complete assay. Compound 194-B was shown by this method to be an effective inhibitor of the histamine flare and it compares favorably with several others tested.

The clinical results obtained in the symptomatic treatment of various allergic conditions with compound 194-B are tabulated (Table I). The group consisted of 249 patients of whom 148 had seasonal hay fever. The dosage in most instances was 50 mg, although a number used 100 mg when sufficient relief was not obtained with the smaller dosage and when side effects were

TABLE I SYMPTOMATIC RELIEF WITH 194 B

ALLERGIC DISEASE	NUMBER OF PATIENTS	SATISFACTORILY HELPED	PER CENT HELPED
Hay fever	148	94	64
Perennial rhinitis	37	15	41
Asthma	30	2	6
Cough (allergic)	12	3	25
Atopic dermatitis	9	3	33
Urticaria	9	3	33
Migraine	3	1	
Heat urticaria	1	0	
Total	249		

not a bar to increased dosage. The frequency and regularity of administration depended upon the character of the complaint. Patients with continuous symptoms, generally those with chronic perennial rhinitis, atopic dermatitis, chronic urticaria, and some with hay fever, took the medication several times daily, usually after meals. The others, whose symptoms were characterized by isolated attacks, used one dose, occasionally repeating it a few hours later if necessary. Careful and repeated observations were carried on to be reasonably certain that the symptomatic relief following medication was due to the treatment.

The best results were obtained by the hay fever patients, with 64 per cent obtaining satisfactory and consistent relief. A smaller percentage of the patients with perennial rhinitis (41 per cent) was benefited. The results



with asthma were poor and similar to what had been our experience with other antihistaminic substances. Because of the small number of the other types of allergic manifestations it is not desirable to draw conclusions until a larger number of patients is treated.

The side effects from compound 194 B were of the same nature as those produced by the older antihistaminic agents. In most instances they were mild and only rarely was it necessary to discontinue medication. Mild sedation was the most common side effect occurring in forty one patients (16 per cent). A few patients complained of nervousness (four), gastrointestinal symptoms (four) and dermatitis (3).

#### SUMMARY

The laboratory experience and clinical results produced by another recently synthesized antihistaminic agent N N dimethyl N 2 thiazolyl N' p methoxybenzyl ethylenediamine hydrochloride (194 B) are reported. This compound was found to have potent antihistaminic and antianaphylactic properties as determined by various experimental methods. Its clinical use was attended with favorable symptomatic relief in seasonal hay fever, but negligible improvement in asthma. Toxic side reactions were few in incidence and mild in degree.

# THE APPRAISAL OF ANTICHLINERGIC ACTIVITY BY PREVENTION OF METHACHOLINE-INDUCED FATAL BRONCHOSPASM IN GUINEA PIGS

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**P**HARMACOLOGICALLY, four etiologic factors are possible in bronchial asthma (A) A local or systemic increase of histamine concentration as in allergy, (B) hyperactivity of the parasympathetic or (C) hypoactivity of the sympathetic nervous system, and (D) toxic agents acting directly on the bronchial muscle, notably the irritants. Accordingly, antihistaminic, anticholinergic, sympathomimetic, and spasmolytic substances have been employed successfully for the relief of asthmatic attacks. Due to their common effect of relaxing the bronchial muscles, all these agents with different pharmacologic actions will alleviate spasm somewhat, regardless of the cause or causes of asthma in question. Therefore it is unreasonable in rational therapy to praise one drug or one type of drug as superior to others in the treatment of bronchial asthma in general. One agent will be more efficacious than others for a particular asthmatic condition for it will specifically antagonize the causative agent or modify a disturbed physiologic function before muscular spasm occurs.

For these reasons we have in the past two years been testing compounds both for their antihistaminic and anticholinergic properties against bronchial spasm in guinea pigs. This will assist us more precisely to elucidate the pharmacodynamics of bronchial dilating drugs in the management of asthmatic conditions. In this paper there will be presented a testing procedure and the anticholinergic activities of a number of substances as determined by this method.

## EXPERIMENTAL

The procedure of Loew, Kaisei, and Moore, based on protection against the lethal effect of histamine aerosol in guinea pigs, was adapted in our work with methacholine. Acetylcholine, which has been employed with or without prostigmine by previous investigators,<sup>1, 2</sup> was found not very satisfactory as a cholinergic stimulant for the bronchial muscles. Acetylcholine in solution is not very stable, furthermore a high concentration of it is required to produce fatal bronchospasm in guinea pigs. Among the stimulants tested, acetyl beta-methylcholine chloride (methacholine) and carbamyl choline chloride were found to kill guinea pigs at very low concentrations in the mist. Owing to its pure muscarinic action and close similarity in other pharmacologic properties to acetylcholine, methacholine was used in our work.<sup>3</sup>

The capacity of the enclosed compartment for the animals is 16 liters, half a cubic centimeter of methacholine solution (1 to 200 dilution) is sprayed

into it in fifteen to twenty seconds. A total of ten minutes is allowed for the animals to breathe the methacholine vapor. The anticholinergic agent is injected intraperitoneally fifteen minutes before the test or, when given orally, thirty minutes beforehand. For each experiment a control is run on animals of the same stock without medication.

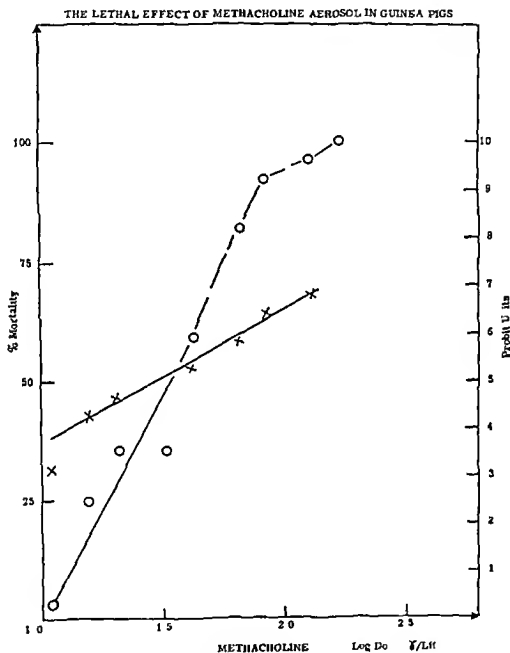


Fig. 1

## RESULTS AND DISCUSSION

In Fig. 1 are given the dose mortality curves with methacholine aerosol in percentages and in probit units. Each point was obtained with twenty-eight animals from a group. One hundred fifty-eight micrograms of methacholine per liter of air (0.5 c.c. of 1 to 200 solution) which would kill around 98 per cent of animals was chosen as a challenging dose for determining the anticholinergic effect of a compound. In Table I are recorded the anticholinergic activities of various compounds some of which possess also antihistaminic or/and spasmolytic properties. The antihistaminic activities of these compounds are given

in the table for comparative studies. The data indicate that atropine is the most effective while aminophylline is the least effective agent for alleviating methacholine-induced bronchospasm in guinea pigs. The results are in good agreement with observations in man from vital capacity determinations.

TABLE I ACTIVITIES OF COMPOUNDS IN PREVENTING FATAL BRONCHOSPASM IN GUINEA PIGS RECEIVING METHACHOLINE OR HISTAMINE AEROSOL

COMPOUND	ANTIMETHACHOLINE PD <sub>50</sub> ± SE* (MG/KG)	ANTIHISTAMINE PD <sub>50</sub> ± SE* (MG/KG)
Atropine sulfate	0.059 ± 0.005	15
Dibutoline sulfate	0.6 ± 0.25	100 (ineffective and toxic)
N-(β-dimethylaminoisopropyl)phenothiazine HCl†	6 ± 1.6	0.3 ± 0.06
2-n-propoxy-4,6-diamino-s-triazine†	15 ± 2.0	15.5 ± 3.5
Diphenhydramine (Benadryl)	25 ± 5	2.1 ± 0.44
Papaverine HCl	29.5 ± 5.7	25
Isonipercaine	64.0 ± 15	7.3 ± 2
Khellin†	75	40
Aminophylline	100	50

\*Intraperitoneal dose average standard error calculated by the method of Miller and Tainter.<sup>4</sup>

†Kindly made available to us by Dr. E. Jones and Dr. C. K. Banks, Chemical Division, Research Laboratories, Parke-Davis & Company.

It is of interest to note the recent publication of Hambourger and associates on the protective action of combinations of diphenhydramine and aminophylline in guinea pigs receiving methacholine aerosol.<sup>6</sup> Their determinations for the individual compounds agree with ours in that large doses of the two are required to obtain a 50 per cent protection of animals inhaling a lethal concentration of methacholine aerosol. Evidently then measurements represent principally the anticholinergic effects of the two substances individually and in combinations. As judged from the antihistamine activities and toxicities of diphenhydramine and aminophylline and then PD<sub>50</sub> antimethacholine doses<sup>6</sup> (Table I), it is improbable that clinically the two substances relieve bronchial asthma chiefly by virtue of their anticholinergic effect. In patients whose asthmatic condition simulates that following methacholine injection,<sup>5,7,8</sup> either atropine or epinephrine should be the therapeutic agent of choice to bring about a quick relief of symptoms.

#### SUMMARY

A procedure is described for evaluating the anticholinergic effect of a compound in fatal bronchospasm of guinea pigs induced with methacholine aerosol.

Of the compounds studied, the decreasing order of potency was as follows: atropine sulfate, Dibutoline sulfate, n-(β-dimethylaminoisopropyl)phenothiazine HCl, 2-n-propoxy-4,6-diamino-s-triazine, diphenhydramine (Benadryl), papaverine, isonipercaine, khellin, and aminophylline.

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in the table for comparative studies. The data indicate that atropine is the most effective while aminophylline is the least effective agent for alleviating methacholine-induced bronchospasm in guinea pigs. The results are in good agreement with observations in man from vital capacity determinations<sup>5</sup>.

TABLE I ACTIVITIES OF COMPOUNDS IN PREVENTING FATAL BRONCHOSPASM IN GUINEA PIGS RECEIVING METHACHOLINE OR HISTAMINE AEROSOL

COMPOUND	ANTIMETHACHOLINE	ANTIHISTAMINE
	PD <sub>50</sub> ± SE* (MG/KG)	PD <sub>50</sub> ± SE* (MG/KG)
Atropine sulfate	0.059 ± 0.005	15
Dibutoline sulfate	0.6 ± 0.25	100 (ineffective and toxic)
N-(β-dimethylaminoisopropyl)phenothiazine HCl†	6 ± 1.6	0.3 ± 0.06
2-n-propoxy-4,6-diamino-s-triazine†	15 ± 2.0	15.5 ± 3.5
Diphenhydramine (Benidryl)	25 ± 5	2.1 ± 0.44
Papaverine HCl	29.5 ± 5.7	25
Isonipercaine	64.0 ± 15	7.3 ± 2
Khellin†	75	40
Aminophylline	100	50

\*Intraperitoneal dose average standard error calculated by the method of Miller and Tainter<sup>4</sup>.

†Kindly made available to us by Dr. E. Jones and Dr. C. K. Banks, Chemical Division, Research Laboratories, Parke, Davis & Company.

It is of interest to note the recent publication of Hamburger and associates on the protective action of combinations of diphenhydramine and aminophylline in guinea pigs receiving methacholine aerosol.<sup>6</sup> Their determination for the individual compounds agree with ours in that large doses of the two are required to obtain a 50 per cent protection of animals inhaling a lethal concentration of methacholine aerosol. Evidently then measurements represent principally the anticholinergic effects of the two substances individually and in combinations. As judged from the antihistaminic activities and toxicities of diphenhydramine and aminophylline and their PD<sub>50</sub> antimethacholine doses<sup>6</sup> (Table I), it is improbable that clinically the two substances relieve bronchial asthma chiefly by virtue of their anticholinergic effect. In patients whose asthmatic condition simulates that following methacholine injection,<sup>5</sup> either atropine or epinephrine should be the therapeutic agent of choice to bring about a quick relief of symptoms.

#### SUMMARY

A procedure is described for evaluating the anticholinergic effect of a compound in fatal bronchospasm of guinea pigs induced with methacholine aerosol.

Of the compounds studied, the decreasing order of potency was as follows: atropine sulfate, Dibutoline sulfate, n-(β-dimethylaminoisopropyl)phenothiazine HCl, 2-n-propoxy-4,6-diamino-s-triazine, diphenhydramine (Benadryl), papaverine, isonipercaine, khellin, and aminophylline.

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TABLE I. HETEROPHILE AGGLUTINATION TITERS IN VARIOUS DIUENTS

PATIENT	STATE OF SERUM	AGGLUTINATION TITERS IN VARIOUS DILUENTS							10% HUMAN GAMMA GLOBULIN
		SALINE	HUMAN SERUM	ASCITIC FLUID	HORSE SERUM	BOVINE SERUM	20% BOVINE ALBUMIN	20% HUMAN ALBUMIN	
<i>Positive Clinical and Hematologic Findings</i>									
HG	Un	1 320	1 5120+	1 5120	1 160	1 320	1 160	1 320	1 160
	Abs	1 80	1 5120	1 320	1 80	1 80	1 40	1 80	1 160
AA	Un	1 640	1 5120+	1 120	1 640	1 1280	1 1280	1 1280	1 320
	Abs	1 320	1 5120+	1 640	1 80	1 80	1 320	1 320	1 160
RS	Un	1 5120	1 10240+	1 10240+	1 2560	1 2560	1 5120	1 5120	1 2560
	Abs	1 640	1 2560	1 1280	1 160	1 160	1 640	1 640	1 320
LW	Un	1 100	1 640	1 640	1 160	1 160	1 320	1 80	1 40
	Abs	1 80	1 160	1 160	1 80	1 160	1 160	1 80	1 40
BH	Un	1 640	1 2560	1 2500	1 320	1 160	1 640	1 320	1 160
	Abs	1 40	1 160	1 160	1 40	1 20	1 40	1 20	1 20
CM	Un	1 640	1 2560	1 5120	1 320	1 640	1 320	-	-
	Abs	1 160	1 640	1 1280	1 80	1 160	1 80	-	-
SS	Un	1 320	1 1280	1 640	1 160	1 320	1 320	1 320	1 320
	Abs	1 160	1 640	1 320	1 80	1 80	1 160	1 160	1 80
<i>Negative Clinical and Hematologic Findings</i>									
BM	Un	1 40	1 320	1 320	1 40	1 40	1 40	1 40	1 20
	Abs	0	0	0	0	0	0	0	0
JG	Un	0	0	0	0	0	0	0	0
	Abs	0	0	0	0	0	0	0	0
AL	Un	1 40	1 160	1 80	1 20	1 20	1 40	1 40	1 20
	Abs	0	0	0	0	0	0	0	0
BG	Un	1 20	1 80	1 40	1 10	1 20	1 20	1 10	1 10
	Abs	0	0	0	0	0	0	0	0
GE	Un	1 10	1 80	1 40	1 10	-	1 10	-	-
	Abs	0	0	0	0	-	0	-	-
Un Unabsorbed									
Abs. Absorbed with guinea pig kidney suspension									

# FURTHER STUDIES ON ENHANCEMENT OF HETEROPHILE AGGLUTINATION TITERS BY MEANS OF SERUM DILUENT

ALBERT MILZER, PH D, AND SHIRLEY NATHAN, BS  
CHICAGO, ILL

RECENTLY we reported that heterophile and various bacterial agglutination titers were significantly enhanced by the use of serum diluent instead of saline<sup>1</sup>. This paper is a further report on the enhancement of heterophile agglutination titers by use of serum and other diluents in place of physiologic saline. Routine heterophile agglutination tests were done, as previously, comparing saline and pooled human serum diluents. Titrations using human ascitic fluid,\* horse serum,\* bovine serum,\* 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent gamma globulin diluents were also carried out.

## METHODS

The technique of Paul and Bunnell<sup>2</sup> was used in heterophile agglutination tests. The patient's serum was first inactivated by heating at 56° C for one half hour. Serial two-fold dilutions were made of the patient's serum in saline diluent, and equal parts of 1 per cent sheep cells in saline were added to each tube. The tubes were shaken and incubated at 37° C for two hours. Next they were removed and kept overnight in the icebox at 5° C. The titer was read as the highest dilution showing distinct visible clumps of erythrocytes.

Tests using ascitic fluid, horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin were carried out in an identical manner except that these various diluents were used in place of saline. Human serum, horse serum, and bovine serum diluents were inactivated by heating at 56° C for one half hour, and all diluents were tested for the presence of heterophile antibodies prior to use. If present, heterophile agglutinins were removed by repeated absorption with sheep cells. Often, heterophile agglutinins were detected in pooled human serum and bovine serum prior to absorption with sheep cells. A diluent and saline corpuscle control tube were included in each test. The Davidsohn guinea pig kidney absorption test for infectious mononucleosis<sup>3</sup> was carried out in a similar manner with the patient's serum absorbed with 20 per cent guinea pig kidney suspension one hour at room temperature prior to titration.

## RESULTS

Heterophile agglutination titrations comparing saline, ascitic fluid, horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin diluents in individuals with positive and negative clinical and hematologic findings for infectious mononucleosis are summarized in Table I. Sheep erythrocytes suspended in saline were added to the tubes containing the various diluents. It was noted that the clumps of sheep cells in the various serum diluents were more easily dispersed on shaking than in saline.

From the Department of Bacteriology and Virology, Medical Research Institute, Michael Reese Hospital.

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TABLE I HETEROPHILE AGGLUTINATION TITERS IN VARIOUS DIUENTS

PATIENT	STATE OF SERUM	AGGLUTINATION TITERS IN VARIOUS DILUTIONS							
		SALINE	HUMAN SERUM	ASCITIC FLUID	HORSE SERUM	BOVINE SERUM	20% BOVINE ALBUMIN	-0% HUMAN ALBUMIN	10% HUMAN OXALMA GLOBULIN
Positive Clinical and Hematologic Findings									
IIG	Un	1 320	1 5120+	1 5120	1 160	1 320	1 160	1 320	1 160
	Abs	1 80	1 5120	1 320	1 80	1 80	1 40	1 80	1 160
AA	Un	1 640	1 5120+	1 5120	1 640	1 1280	1 1280	1 1280	1 320
	Abs	1 320	1 5120+	1 640	1 80	1 80	1 320	1 320	1 160
RS	Un	1 5120	1 10240+	1 10240+	1 2560	1 2560	1 5120	1 5120	1 2560
	Abs	1 640	1 2560	1 1280	1 160	1 160	1 640	1 640	1 320
RW	Un	1 160	1 640	1 640	1 160	1 160	1 320	1 80	1 40
	Abs	1 80	1 160	1 160	1 80	1 160	1 160	1 80	1 40
BH	Un	1 640	1 2560	1 2560	1 320	1 160	1 640	1 320	1 160
	Abs	1 40	1 160	1 160	1 40	1 40	1 40	1 40	1 20
CM	Un	1 640	1 2560	1 5120	1 320	1 640	1 320	-	-
	Abs	1 160	1 640	1 1280	1 80	1 160	1 80	-	-
SS	Un	1 320	1 180	1 640	1 160	1 320	1 320	1 320	1 320
	Abs	1 160	1 640	1 320	1 80	1 80	1 160	1 160	1 80
Negative Clinical and Hematologic Findings									
BM	Un	1 40	1 320	1 320	1 40	1 80	1 40	1 40	1 20
	Abs	0	0	0	0	0	0	0	0
JG	Un	0	0	0	0	0	0	0	0
	Abs	0	0	0	0	0	0	0	0
AL	Un	1 40	1 160	1 80	1 20	1 40	1 40	1 40	1 20
	Abs	0	0	0	0	0	0	0	0
BG	Un	1 20	1 80	1 40	1 10	1 20	1 20	1 20	1 10
	Abs	0	0	0	0	0	0	0	0
CE	Un	1 10	1 80	1 40	1 10	-	1 10	-	-
	Abs	0	0	0	0	-	0	-	-
Un Unabsorbed Abs. Absorbed with guinea pig kidney suspension									

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ALBERT MILZER, PH D , AND SHIRLEY NATHAN, BS  
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RECENTLY we reported that heterophile and various bacterial agglutination titers were significantly enhanced by the use of serum diluent instead of saline<sup>1</sup>. This paper is a further report on the enhancement of heterophile agglutination titers by use of serum and other diluents in place of physiologic saline. Routine heterophile agglutination tests were done, as previously, comparing saline and pooled human serum diluents. Titrations using human ascitic fluid,<sup>2</sup> horse serum,<sup>3</sup> bovine serum,<sup>4</sup> 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent gamma globulin diluents were also carried out.

## METHODS

The technique of Paul and Bunnell<sup>5</sup> was used in heterophile agglutination tests. The patient's serum was first inactivated by heating at 56° C for one half hour. Serial two-fold dilutions were made of the patient's serum in saline diluent, and equal parts of 1 per cent sheep cells in saline were added to each tube. The tubes were shaken and incubated at 37° C for two hours. Next they were removed and kept overnight in the icebox at 5° C. The titer was read as the highest dilution showing distinct visible clumps of erythrocyte.

Tests using ascitic fluid, horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin were carried out in an identical manner except that these various diluents were used in place of saline. Human serum, horse serum, and bovine serum diluents were inactivated by heating at 56° C for one half hour, and all diluents were tested for the presence of heterophile antibodies prior to use. If present, heterophile agglutinins were removed by repeated absorption with sheep cells. Often, heterophile agglutinins were detected in pooled human serum and bovine serum prior to absorption with sheep cells. A diluent and saline corpuscle control tube were included in each test. The Dravidsohn guinea pig kidney absorption test for infectious mononucleosis<sup>6</sup> was carried out in a similar manner with the patient's serum absorbed with 20 per cent guinea pig kidney suspension one hour at room temperature prior to titration.

## RESULTS

Heterophile agglutination titrations comparing saline, ascitic fluid, horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin and 10 per cent human gamma globulin diluents in individuals with positive and negative clinical and hematologic† findings for infectious mononucleosis are summarized in Table I. Sheep erythrocytes suspended in saline were added to the tubes containing the various diluents. It was noted that the clumps of sheep cells in the various serum diluents were more easily dispersed on shaking than in saline.

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## LABORATORY METHODS

### A COMPARISON OF EOSIN ACETONE AND PHLOXINE PROPYLENE GLYCOL DILUENTS IN EOSINOPHIL COUNTS

CAPTAIN PHILIP H. HENNEMAN, M.C. \* HILDA WYLLER, A.B. AND  
MARY M. WESTENHAYER  
WASHINGTON, D. C.

THE potential value of direct chamber counts of circulating eosinophils as an adjunct in studies of adrenal activity has been demonstrated by Thorn.<sup>1</sup> Randolph examined previous methods for direct eosinophil counts in 1944 and experimented with several diluents including certain ones with a propylene glycol base. He concluded that diluents consisting of eosin, acetone and water were unsatisfactory because of lysis of leucocytes in these fluids. The present study was designed to evaluate two diluents for eosinophil counts.

The diluent used by Thorn<sup>1</sup> and a modification of Randolph's eosinophil diluent were prepared as follows:

EOSIN ACETONE DILUENT		PHLOXINE PROPYLENE GLYCOL DILUENT	
Aqueous eosin 2 per cent	50 cc	Phloxine	0.05 Gm
Acetone	50 cc	Propylene glycol	50.0 cc
Distilled water	90.0 cc	Distilled water	50.0 cc

#### METHODS

Freshly drawn venous blood was placed in bottles containing sufficient oxalate to prevent clotting. Twenty-four white cell pipettes were filled at once with blood to the 1.0 mark. Twelve of these pipettes were filled to the 1.1 mark with the eosin acetone diluent and twelve with the phloxine propylene glycol diluent. After three, eighteen and seventy-eight minutes respectively, four pipettes containing the eosin acetone diluent and four containing the phloxine propylene glycol diluent were placed in a mechanical shaker for two minutes and the eosinophils promptly counted. Thus the effect of continued contact of the blood with each diluent for five, twenty, and eighty minutes was observed. Identical studies were performed after the same oxalated blood had aged two and four hours. The oxalated blood was mixed thoroughly by gentle agitation before sampling. Both sides of a standard Spencer Bright Line counting chamber were filled from each pipette and all the eosinophils within the ruled areas counted. Thus 18/10 cubic millimeters of blood diluted 1:10 were examined.

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Since the phloxine-propylene glycol diluent required fifteen minutes for complete staining of the eosinophils, the blood counting chambers were filled at the five-minute periods as with the eosin-acetone method, but the actual counting was delayed until fifteen minutes of staining time had elapsed

### RESULTS

The calculated eosinophil counts performed with the two diluents are listed in Table I and diagrammed in Figs 1 and 2

TABLE I

EOSINOPHIL COUNTS WITH EOSIN ACETONE DILUENT									
TIME AFTER DILUTION OF BLOOD	5 MIN			20 MIN			80 MIN		
AGE OF BLOOD	0 HR	2 HR	4 HR	0 HR	2 HR	4 HR	0 HR	2 HR	4 HR
Sample 1	161	161	222	66	33	44	44	5	16
Sample 2	216	127	200	88	38	11	22	5	22
Sample 3	177	138	177	50	50	38	11	11	0
Sample 4	156	172	150	44	27	16	27	11	5
Mean	178	150	187	62	37	27	26	8	11

EOSINOPHIL COUNTS WITH PHLOXINE PROPYLENE GLYCOL DILUENT									
TIME AFTER DILUTION OF BLOOD	5 MIN			20 MIN			80 MIN		
AGE OF BLOOD	0 HR	2 HR	4 HR	0 HR	2 HR	4 HR	0 HR	2 HR	4 HR
Sample 1	177	244	166	138	216	144	227	172	266
Sample 2	222	172	200	194	183	211	211	177	272
Sample 3	189	255	166	194	266	133	177	205	244
Sample 4	156	216	233	166	238	205	244	161	144
Mean	186	222	193	173	226	173	215	179	232

The eosin-acetone diluted blood showed a striking decrease in cell count with lapse of time after dilution of the blood. Using this diluent there was also a marked decrease in cell count with increasing age of the ovalated blood. The phloxine-propylene glycol method showed no significant change in count with lapse of time after dilution of the blood nor with aging of the ovalated blood.

The decrease in count with lapse of time after dilution of the blood with the eosin-acetone fluid was highly significant statistically ( $p < 0.001$ ) as determined by analysis of variance. The analysis further revealed that the nature of this decrease was complex. The decrease in eosinophil counts obtained with the aged blood was also highly significant ( $p < 0.01$ ). The relationship of count to age of blood was curvilinear. These results suggest that multiple factors are involved in these decreases.

Similar analysis of the values obtained with the phloxine-propylene glycol diluent revealed no significant change with time or age of blood. Hence all the phloxine-propylene glycol counts were pooled, providing a mean count of 199 eosinophils per cubic millimeter of blood with a standard deviation of 36 cells or 18 per cent. This emphasizes the large inherent error in this type of determination.

Since Randolph<sup>2</sup> observed the lysis of leucocytes in aqueous acetone diluents, the following additional study was undertaken in this laboratory. Blood was diluted with the eosin acetone fluid and placed in a counting chamber with a thin cover slip and sealed with petroleum jelly. There was no change in the eosinophil count over a period of three hours. However, immediate examination of the cells under high magnification revealed the forma-

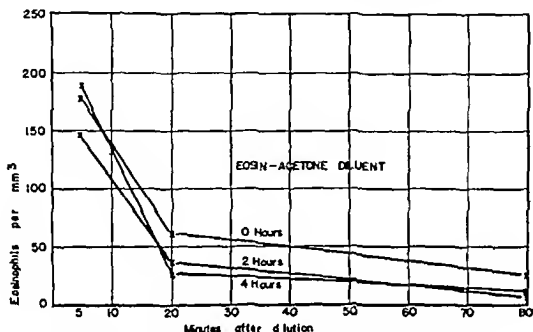


Fig. 1—Circulating eosinophil counts performed at three time intervals showing change in counts with passage of time and aging of oxalated blood

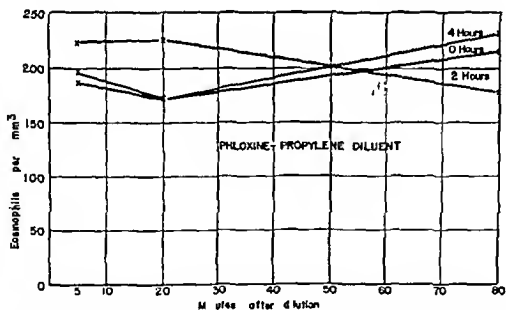


Fig. 2—Circulating eosinophil count performed at three time intervals after dilution showing lack of significant change in counts with passage of time and aging of oxalated blood

tion of blebs on the limiting membrane of the eosinophils followed by rupture of the cells. If the preparation was not agitated, the individual aggregates of eosinophilic granules could still be counted as eosinophils even though the cell membranes were disrupted. If the pipette containing blood and the eosin acetone diluent was agitated after allowing several minutes for lysis of cells the granules were scattered and there was an apparent decrease in eosinophil count.

Since the phloxine-propylene glycol diluent required fifteen minutes for complete staining of the eosinophils, the blood counting chambers were filled at the five-minute periods as with the eosin-acetone method, but the actual counting was delayed until fifteen minutes of staining time had elapsed

### RESULTS

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Sample 2	216	127	200	88	38	11	22	5	22
Sample 3	177	138	177	50	50	38	11	11	0
Sample 4	156	172	150	44	27	16	27	11	5
Mean	178	150	187	62	37	27	26	8	11

EOSINOPHIL COUNTS WITH PHLOXINE PROPYLENE GLYCOL DILUENT									
TIME AFTER DILUTION OF BLOOD	5 MIN			20 MIN			80 MIN		
AGE OF BLOOD	0 HR	2 HR	4 HR	0 HR	2 HR	4 HR	0 HR	2 HR	4 HR
Sample 1	177	244	166	138	216	144	237	172	266
Sample 2	222	172	200	194	183	211	211	177	272
Sample 3	189	255	166	194	266	133	177	205	244
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## A SIMPLE METHOD FOR ASEPTIC GRINDING OF SMALL AMOUNTS OF TISSUE

F J MURRAY, PH D  
CINCINNATI OHIO

DURING the course of routine passages of mouse encephalitis viruses it was necessary to grind individual brains. The usual method for such grinding involves the use of a small pestle and mortar, previously wrapped in paper and sterilized. Where many pestles and mortars are required a considerable saving of space may be effected by use of the method here described. Probably the greatest disadvantages of using pestle and mortar are the possibilities of contamination of the material being ground and the possibility of infection of the person doing the grinding.

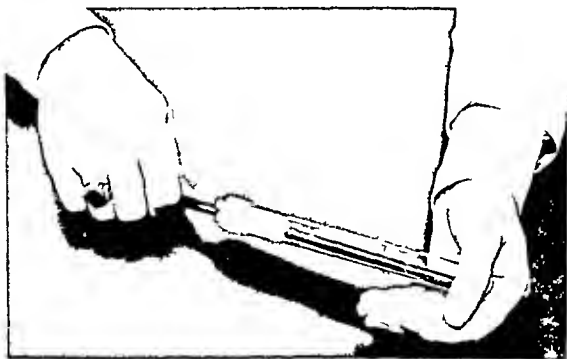


Fig. 1—Assembly for grinding tissues

The method employed by me calls for 6 in. Pyrex test tubes with 10 in. glass rods fitted in the tubes through the centers of the cotton stoppers. The tube-rod assemblies are sterilized in the autoclave and dried in the oven. Sterile aluminum or other suitable grinding material may be added at the time the mouse brain is placed in the tube or it may be added beforehand and sterilized with the assembly.

From the Bacteriology Department Research Laboratories the Wm. S. Merrell Company  
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When the brain and diluent have been added, the tube is held loosely in the palm of the left hand, with the thumb stretched across the bottom of the tube (Fig 1) The portion of the rod showing above the cotton plug is grasped by the right hand and is made to perform a gentle whipping motion The angle of the tube, position of the hands, etc , may be varied to the comfort of the individual employing the technique

Obviously this technique is not limited to the grinding of brains, but may be used for lungs and other animal tissues



## PRESERVATION OF VIRUSES IN A MECHANICAL REFRIGERATOR AT $-25^{\circ}\text{C}$

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HAROLD S. GINSBERG, M.D. AND FRANK L. HORSFALL, JR., M.D.  
NEW YORK, N. Y.

AS A routine, many laboratory workers who employ viruses in investigations or in diagnostic procedures store infectious material in the frozen state at about  $-70^{\circ}\text{C}$  in cabinets<sup>1</sup> that are refrigerated with solid carbon dioxide. Although this way of preservation is useful and effective, it has some disadvantages. Of these, the most important are (1) the necessity for a continuous supply of solid  $\text{CO}_2$ , (2) the high annual cost of solid  $\text{CO}_2$ , and (3) the acidity caused by the diffusion of  $\text{CO}_2$  into specimens. In many geographical areas a regular supply of solid  $\text{CO}_2$  cannot be obtained. In areas where the refrigerant is readily available, the cost of constantly maintaining low temperatures with solid  $\text{CO}_2$  is considerable even when efficient cabinets are employed (the outlay may run to \$100 to \$200 per cubic foot of storage space per year). Although diffusion of  $\text{CO}_2$  into specimens can be prevented by the use of sealed hard glass containers this procedure is cumbersome and inconvenient.

Subfreezing temperatures now can be maintained with a number of commercially available mechanical refrigerators. Certain electrically operated ones maintain temperatures in the range attained with cabinets cooled with solid  $\text{CO}_2$  but their initial cost is high and their storage capacity not large. Other less costly refrigerators can maintain temperatures in the range of  $-20$  to  $-30^{\circ}\text{C}$  and of these many provide large storage space.

The preservation of viruses for prolonged periods at  $-70^{\circ}\text{C}$  has been studied in a number of instances<sup>1-3</sup> and it is now well known that most viruses retain infectivity for long intervals when properly stored at this temperature. However, relatively little is known as to the stability of viruses at  $-20$  to  $-30^{\circ}\text{C}$ . Melnick<sup>4</sup> found that Y-SK and Ph-phomyelitis viruses as well as the TO Theiler virus retained their original titers when stored for twelve months at  $-20^{\circ}\text{C}$ . Under the same conditions the titer of Japanese B virus decreased by one thousand fold or more.

In the present investigation infectivity titrations were carried out with a number of different viruses which were stored in an electrically operated mechanical refrigerator for periods up to twelve months at temperatures ranging from  $-20$  to  $-30^{\circ}\text{C}$ . The results indicate that all of the viruses studied can be stored under these conditions for at least three months without causing any striking reduction in infectivity. With most of the neurotropic viruses full infectivity was maintained for nine months. With the influenza mumps group of viruses infectivity was much diminished although still present after twelve

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months It should be mentioned that during the study one refrigerator was once accidentally disconnected, and the other twice failed to operate properly During these periods the temperature increased sufficiently so that all specimens were thawed for an interval of less than twenty-four hours

#### EXPERIMENTAL

The following viruses were employed Eastern equine encephalitis (Rockefeller Institute strain), Western equine encephalitis (Rockefeller Institute strain), Russian Far East encephalitis (No 1 strain), Japanese B encephalitis (Nakayama strain), poliomyelitis (Lansing strain) and poliomyelitis (MEF1 strain), influenza A virus (PR8 strain), influenza B virus (Lee strain), and mumps virus (Habel strain)

The neurotropic viruses were contained in suspensions of fresh, infected mouse brains or in frozen suspensions previously stored in a CO<sub>2</sub> cabinet The suspensions stored were 10<sup>-1</sup> dilutions of brain in either 10 or 50 per cent normal rabbit serum which were placed either in screw-capped nitrocellulose tubes or in sealed Pyrex ampules Influenza and mumps viruses were contained in allantoic fluid obtained from infected chick embryos One portion of each allantoic fluid was stored in the undiluted state, the other after dilution to 10<sup>-1</sup> with normal horse serum The allantoic fluids were all kept in screw-capped nitrocellulose tubes

In every instance a number of specimens of each virus preparation were quickly frozen by partial submersion in a mixture of ethyl alcohol and solid CO before they were placed in the refrigerator at -20 to -30° C At appropriate intervals one specimen of each frozen suspension was thawed rapidly in running tap water and an infectivity titration was performed Titrations were carried out with serial decimal dilutions With the neurotropic viruses the usual intra cerebral technique in white mice was employed, and the LD<sub>50</sub> was determined In the case of influenza and mumps viruses the intra-allantoic technique in chick embryos was used, and the EID<sub>50</sub> was determined by hemagglutination tests with allantoic fluids removed after an appropriate interval

The refrigerators employed were commercially available models which had single stage compressor units and were capable of maintaining temperatures of -30° C on intermittent operation One refrigerator had a storage capacity of 20 cubic feet, the other, 16

The results of the titrations are presented in Table I It will be noted that all of the viruses which were suspended in 50 or more per cent normal serum retained infectivity throughout the entire period of observation It will also be seen that the Russian Far East encephalitis virus and the MEF1 and the Lansing strains of poliomyelitis virus retained almost their original LD<sub>50</sub> titer for a period of nine months Melnick<sup>4</sup> also showed a similar relative stability for other Lansing-type viruses The Western equine encephalitis virus showed only a slight reduction in titer after nine months of storage and the titer of the Eastern equine encephalitis virus decreased only about 1.5 log units over the same period However, the Japanese B encephalitis virus, although still infective (i.e., LD<sub>50</sub> 10<sup>2.6</sup>) after nine months, had decreased markedly in titer This latter finding is in good agreement with the results reported by Melnick<sup>4</sup>

TABLE I EFFECT OF STORAGE AT -20 TO -30° C ON INFECTIVITY OF VIRUSES

VIRUS	MATERIAL USED	SUSPENDED IN	ORIGINAL TITER LD <sub>50</sub>	TITER AFTER STORAGE		
				3 MO	6 MO	9 MO
				LD <sub>50</sub>		
Eastern equine	10 <sup>1</sup> m b	50% nrs	10 <sup>9.0</sup>	10 <sup>7.4</sup>	10 <sup>7.2</sup>	10 <sup>7.5</sup>
Western equine	10 <sup>1</sup> m b	50% nrs	10 <sup>8.3</sup>	10 <sup>8.0</sup>	10 <sup>7.8</sup>	10 <sup>7.8</sup>
Russian Far East	10 <sup>1</sup> m b (fresh)	10% nrs	10 <sup>8.8</sup>	10 <sup>5</sup>	10 <sup>3.0</sup>	—
Russian Far East	10 <sup>1</sup> m b (fresh)	50% nrs	10 <sup>9.4</sup>	10 <sup>9.4</sup>	10 <sup>8.8</sup>	10 <sup>8.6</sup>
Japanese B	10 <sup>1</sup> m b (fresh)	50% nrs	10 <sup>8</sup>	10 <sup>7</sup>	10 <sup>4</sup>	10 <sup>0</sup>
Pohomyelitis Lansing	10 <sup>1</sup> m b	50% nrs	10 <sup>2</sup>	10 <sup>2.1</sup>	10 <sup>2</sup>	10 <sup>2.2</sup>
Pohomyelitis, MEF1	10 <sup>1</sup> m b (fresh)	50% nrs	10 <sup>3.2</sup>	10 <sup>2.9</sup>	10 <sup>3</sup>	10 <sup>3.3</sup>
				EID <sub>50</sub>		
				3 MO	6 MO	1 YR
Influenza A PR8	10 <sup>1</sup> all fl	90% nhs	10 <sup>8.8</sup>	10 <sup>8.4</sup>	10 <sup>5.0</sup>	10 <sup>5.0</sup>
Influenza A PR8	Undil all fl	—	10 <sup>8.3</sup>	10 <sup>7</sup>	10 <sup>4.7</sup>	<10 <sup>3.0</sup>
Influenza B Lee	10 <sup>1</sup> all fl	90% nhs	10 <sup>7.8</sup>	10 <sup>7.0</sup>	10 <sup>4.5</sup>	10 <sup>3.3</sup>
Influenza B Lee	Undil all fl	—	10 <sup>7.8</sup>	10 <sup>7.0</sup>	10 <sup>3.5</sup>	<10 <sup>0</sup>
Mumps	10 <sup>1</sup> all fl	90% nhs	10 <sup>7</sup>	10 <sup>7.0</sup>	—	10 <sup>4</sup>
Mumps	Undil all fl	—	10 <sup>6.4</sup>	10 <sup>7</sup>	—	<10 <sup>1.0</sup>

m b Mouse brain suspension in normal rabbit serum

nrs Normal rabbit serum in saline solution

all fl Allantoic fluid

nhs Normal horse serum

EID<sub>50</sub> 50 per cent end point of virus titration in chick embryos

It will be observed that influenza A, influenza B, and mumps viruses showed only slightly reduced EID<sub>50</sub> titers after storage for three months. However, after six months of storage the titers of the influenza viruses had decreased by more than 3 log units. This was true even in the case of the suspensions which had been prepared in 90 per cent normal horse serum. After storage for one year none of the three viruses kept in undiluted allantoic fluid showed any evidence of infectivity and all suspended in horse serum gave titers 3 to 4 log units lower than the original titers of the suspensions.

#### DISCUSSION

It appears evident that a number of neurotropic viruses can be preserved by storage at -20 to -30° C for periods of at least nine months without much loss of infectivity. Japanese B encephalitis virus seems to be the least stable of the neurotropic viruses tested; it showed a marked reduction in titer after storage for six months at this temperature. Influenza and mumps viruses exhibited a stability under the conditions employed which was approximately of the same order as that of Japanese B virus. Their infectivity titers also were considerably reduced following storage for six months at -25° C.

It should be emphasized that of the viruses employed those suspended in 50 to 90 per cent normal animal serum retained infectivity sufficient to permit recovery easily after storage for at least nine months. Moreover, when such concentrations of normal serum were used none of the virus suspensions showed any striking reduction in titer after three months of storage; only those of Eastern equine and Japanese B encephalitis exhibited a decrease of 1 or more log units. It also should be stressed that the results were obtained despite the fact that the viruses had been accidentally thawed once or twice for an interval of some hours.

For many virus studies a period of storage of three months is satisfactory. In laboratories in which a CO<sub>2</sub> refrigerated cabinet is not available, and in areas in which a supply of solid CO<sub>2</sub> cannot be obtained, electrically operated mechanical refrigerators which maintain a temperature of -20 to -30° C may prove useful.

#### SUMMARY

Numerous viruses can be preserved by storage in an electrically operated mechanical refrigerator at -20 to -30° C. The infectivity titer of a number of viruses did not diminish significantly after storage for three months. In spite of the fact that some showed a marked reduction in titer after nine months, none became inactivated if stored in the presence of an adequate amount of normal serum.

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## A MACERATOR FOR SMALL SAMPLES OF TISSUE

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IT IS often necessary to macerate small samples of fresh tissues for analysis before extraction with solvents. Grinding in a mortar involves transfer with an increase in the volume of washings and the risk of small losses. The Waring Blender is an excellent instrument for the maceration of fresh tissues, but transfer to other vessels is necessary and is sometimes troublesome when the samples are small.

We have devised the highly efficient macerator, shown in Fig. 1, whose cutting blades are small enough to be inserted into a large sized test tube. Samples of tissue are macerated in the extraction vessel, thus obviating a transfer and increasing speed and accuracy. The instrument processes organs and tissue samples of 2 to 25 grams and, if suitably modified, much larger samples could be finely macerated.

The gear housing (A) is made from  $\frac{1}{4}$  in. by  $\frac{3}{4}$  in. brass stock bent to form a rectangle with inside dimensions  $1\frac{1}{2}$  in. by  $2\frac{1}{4}$  in. Cover plates of copper sheeting are made to match the open sides. Lubricating grease covers the inside of the housing. The mounting bar (B) is of  $\frac{3}{8}$  in. diameter brass rod 7 in. long, tapped into an end of the housing. Shaft (C) for the driving gear is coupled to an electric motor by a short length of pressure tubing. A General variable speed stirrer (80 to 1300 r.p.m.) is suitable for the purpose in the upper speed range.

The driving gear actuates gears  $D_1$  and  $D_2$ , counterrotating the  $\frac{1}{8}$  in. diameter by  $8\frac{1}{2}$  in. stainless steel spindles centered  $\frac{3}{8}$  in. apart. There is a step up from the driving to the driven gears of 3 to 1, the speed of the two spindles being equal. The cutter spindles are supported by a bearing (E), which is  $\frac{1}{2}$  in. above the cutter blades and is mounted on a stainless steel rod support (F)  $\frac{1}{4}$  in. diameter and  $4\frac{1}{2}$  in. long, tapped into the bottom of the housing. The cutters,  $G_1$  and  $G_2$ , 37 mm. by 11.5 mm., are formed from 24 gauge stainless steel in a modified "S" shape with cutting edges. A curved plate (H) mounted on  $\frac{1}{8}$  in. diameter rod is supported from the stand by a clamp and serves as a guide for a test tube.

The cutting blades mesh when rotated giving fine maceration. The tendency to vortex formation by one blade is nullified by the counterrotation of the other so that fluid does not mount the sides of the vessel. The long length of the blades helps to prevent the trapping of tissue above the cutters.

The instrument is simple to operate. In this laboratory it is used to macerate rat livers for the determination of total lipids. A 28 mm. by 150 mm. Pyrex test tube containing a liver (approximately 8 to 10 grams) with sufficient acetone

to cover the organ is placed so that the cutting blades are inserted to about 1 m above the solvent and the test tube is held against its guide. The motor is turned on and the vessel is slowly raised against the guide until the cutters enter the solvent and end near the bottom of the tube. Maceration is complete within one



Fig 1

minute. The tube is partially withdrawn, the motor immediately switched off, and the cutters are quickly washed with a stream of acetone from a wash bottle. After removal of the supernatant fluid the liver may be re-extracted directly in the tube by fresh solvents.

With the instrument shown, the macerating cutters can be inserted into a vessel as small as 23 mm in diameter, but it is obvious that the size and form of the cutters may be modified to suit many types of vessels and tissues or materials requiring maceration.

We wish to acknowledge the assistance of Mr J D Brown, Mr E B Johnson, and Mr J H Royce in the development of the instrument.

## THE EFFECT OF RIGID SODIUM RESTRICTION IN PATIENTS WITH CIRRHOSIS OF THE LIVER AND ASCITES

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LOW salt diets have been used for many years in the treatment of edema and ascites in patients with cirrhosis of the liver. Enthusiastic reports appeared in the French literature as early as 1901 claiming actual termination of ascites formation from the use of a low salt diet.<sup>1, 2, 3</sup> In recent years, however, emphasis on high protein high caloric diets in treatment of cirrhosis has led many physicians away from the use of low salt regimens since it has seemed difficult to devise a palatable low salt diet which is adequate in protein. Moderate restriction of salt has continued to be common practice in the treatment of ascites but results have not been striking.

Recently numerous reports have appeared dealing with the role of sodium in the formation of ascites and edema in cardiac disease.<sup>4, 5, 6</sup> Sodium and water retention by the kidney has been claimed as a factor in the production of ascites in cirrhosis as well as in congestive heart failure by Farnsworth.<sup>7</sup> Layne and Schemm<sup>8</sup> have applied this principle to the management of cardiac and cirrhotic ascites by means of low salt diets. Chalmers and Davidson<sup>9</sup> have shown in a preliminary study of sodium balance in cirrhosis that salt plays an important role in fluid retention. Recently the work of Whipple and co-workers<sup>10</sup> on dogs rendered ascitic by constriction of the inferior vena cava above the liver has demonstrated that ascites of purely mechanical origin can be affected markedly by variations in salt intake.

The role of hypoalbuminemia in the formation of ascites in patients with liver disease has been recognized by numerous observers.<sup>11, 12</sup> Ralli and co-workers<sup>13</sup> have obtained evidence that increased excretion of antidiuretic substance also plays a role in ascites formation. Recently, the work of Blake more<sup>14</sup> has demonstrated the importance of portal hypertension as a third factor in the formation of ascites. The present report represents a description of the direct relationship between the sodium content of the diet and the accumulation of ascites in thirteen patients despite the operation of the other three factors. Detailed studies of electrolyte balance in three of these patients will be published in a separate communication.

### MATERIALS AND METHODS

The thirteen patients to be discussed were admitted to the Hospital of The Rockefeller Institute at least ten days prior to the initiation of salt restriction in the diet. The diagnosis

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of cirrhosis of the liver was established by characteristic history, numerous physical examinations, x-ray studies of the esophagus and abdomen, and a wide variety of liver function tests. Ascites had been present for four to forty eight months prior to the onset of low salt therapy and had necessitated two to seventy eight paracenteses. Fluid continued to accumulate at the usual rate at the time of onset of therapy.

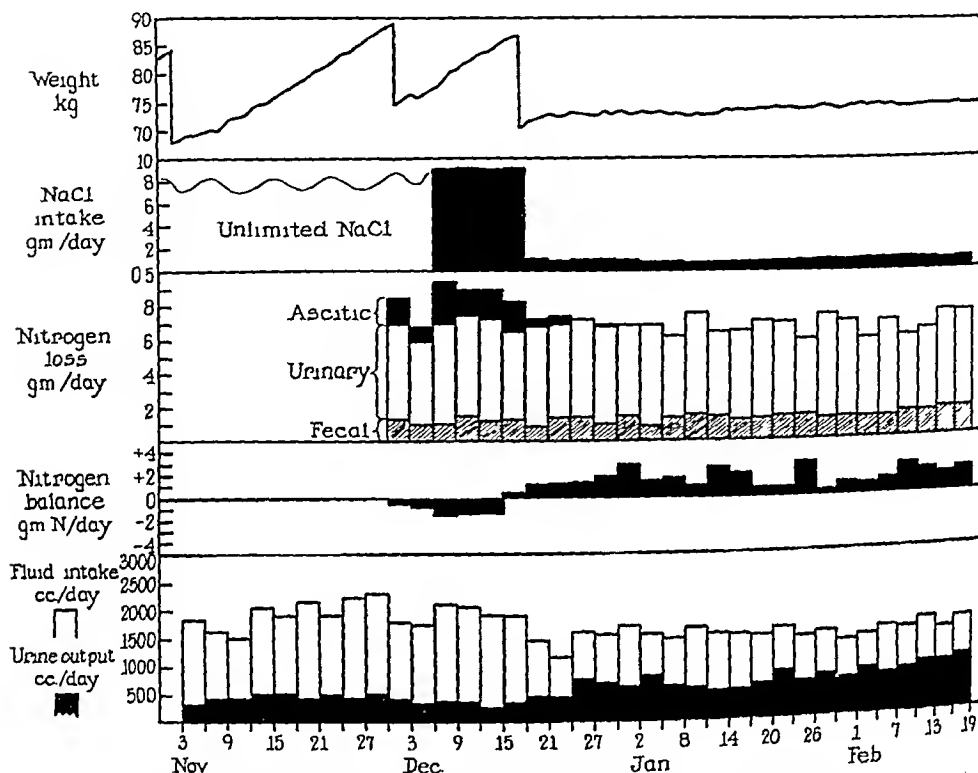


Fig 1—Case 1. Three paracenteses in the hospital prior to Na restriction. Immediate cessation of ascites formation on diet of 1 Gm of NaCl per day. Reversion from negative to positive nitrogen balance.

The etiology of the cirrhosis was chronic alcoholism with associated nutritional disturbance in nine of the thirteen patients. None of the patients was suffering from an enlarged, acutely decompensated fatty liver at the time of admission.

The diets used were prepared by trained dietitians who kept careful records of the daily intake of protein, fat, carbohydrate, and salt. An effort was made to provide patients with an excess of food and calculations were made from the amounts offered and refused. Protein, salt, and calorie intake were calculated from standard tables. The sodium intake was checked by direct analyses. In the detailed studies on sodium balance, direct analysis of the sodium content of the food, urine, and feces was carried out (NaCl was calculated on the basis of measurements of Na). In the low salt diets, special attempts were made to keep the food palatable. Salt free bread, available for purchase, was used. No salt substitutes were administered. Where protein intake tended to be low, or where a high protein, low sodium intake was sought, whole protein supplements were added to the diet.\* As much as 50 Gm of protein per day could be provided with these palatable supplements. Serum albumin was determined by a modification of the specific immunologic method of Chow<sup>12</sup>. Instead of measuring turbidity, the antigen antibody precipitate was determined by means of the molybdenum reagent of Moore and Stein<sup>13</sup>. The Howe method with Kjeldahl digestion

\*Delcos Granules (Sharp & Dohme) Melactin (Squibb) Protinal (National Drugs)



was also used for protein determination. Osmotic pressures were determined by a specially constructed Hepp type osmometer. Determinations of serum bilirubin, bromsulfalein retention, thymol turbidity and total lipids were also carried out in all of the patients during the period of therapy. The methods employed were identical with those presented in a previous study.<sup>17</sup> Plasma chlorides were determined by the method of Van Slyke<sup>18</sup>, plasma volume, by the method of Gibson and Evans.<sup>19</sup> Sodium was determined by means of a Perkin Elmer flame photometer utilizing lithium as an internal standard.

### RESULTS

**CASE 1**—A 52 year old man with cirrhosis of unknown etiology beginning six months prior to admission and requiring six paracenteses. Physical examination showed occasional spider angiomas and a small hard liver. Laboratory tests indicated an advanced cirrhosis. The total protein was 5.7 Gm per cent with albumin 1.9 Gm per cent. Bromsulfalein retention was 37 per cent in 45 minutes. Serum esterase was 22 units. Cephalin flocculation was 2+ the thymol turbidity was 10 units and bilirubin was 1.8 mg per cent.

On admission the patient continued to accumulate ascites at a regular rate (Fig 1), necessitating three paracenteses while on an unlimited NaCl intake. This was found by analysis to average 9.2 Gm NaCl per day. Urinary excretion of NaCl was extremely low, averaging 0.04 Gm per day while fecal NaCl was 0.3 Gm per day. The patient was in negative nitrogen balance on the normal diet. This was chiefly due to the loss of ascitic nitrogen through the paracenteses.

Immediately following the initiation of a low salt diet which on analysis was found to contain between 0.8 and 1.2 Gm NaCl per day, the ascites formation ceased and the patient reverted to positive nitrogen balance as indicated in Fig 1. The urinary output approximately doubled and both the urinary and fecal NaCl concentrations fell. The serum and ascitic fluid Na levels showed a fall of approximately 3 meq per liter. After the patient remained on this diet for three months the urinary NaCl which had remained constant suddenly increased and the patient lost considerable ascites. Table I illustrates the sudden rise in the urinary NaCl despite continued NaCl restriction. The dietary intake improved progressively during this time and positive nitrogen balance increased. At the present time, six months after the initiation of Na restriction the patient is markedly improved although not entirely recovered. He now tolerates 4 and 5 Gm NaCl per day without forming ascites because of the increased urinary output of Na.

The total protein in the serum and ascitic fluid rose by 2 Gm per cent after five months on the diet.

TABLE I

DATE	NaCl GM/DAY			Na MEQ/LITER	
	FOOD	URINE	FECES	SERUM	ASCITIC FLUID
Dec 3	9.1	0.05	0.30	138	137
Dec 15	9.3	0.07	0.35	139	139
Jan 12	1.1	0.02	0.15	135	135
Feb 12	0.9	0.02	0.12	135	134
Mar 24	1.0	2.00	0.25	137	137
May 12	0.9	1.00	0.13	136	135

**Comment**—Rigid NaCl restriction caused a cessation of ascites formation, positive nitrogen balance and a rise in serum and ascitic fluid proteins. Urinary NaCl increased with the mobilization of ascitic fluid. A five month period of therapy was necessary before the patient showed signs of recovery.

**CASE 2**—Fig 2 illustrates the course of a 42 year old woman with a nutritional cirrhosis who had ascites for eight months prior to admission. She had a total of twenty nine paracenteses in that period, during which time she was at home on minimal activity. Since an

accurate record of these was kept, they are indicated in the weight curve of Fig 2 Her diet, poor at the onset of the ascites, was adequate for six months prior to admission The skin showed numerous spider angiomas and prominent liver palms The liver edge was barely palpable at the costal margin An esophagram showed esophageal varices Laboratory tests likewise pointed to an advanced cirrhosis Proteins formed by the liver were all low, plasma albumin was 2.4 Gm per cent, fibrinogen 225 mg per cent, prothrombin

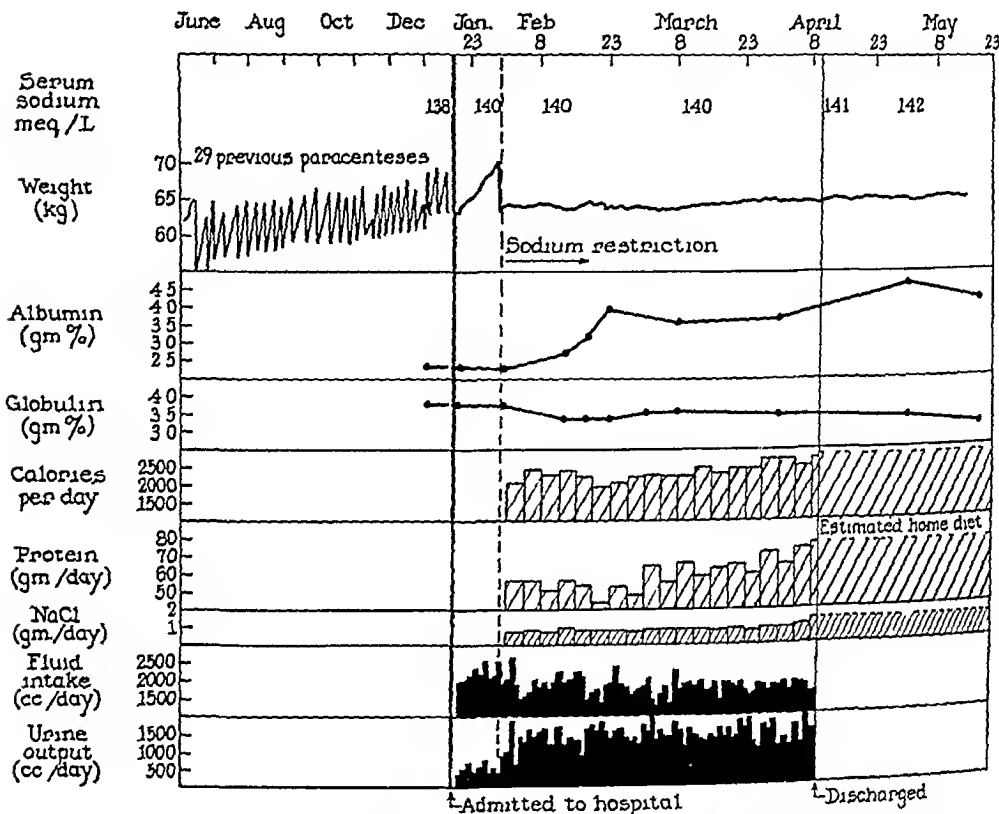


Fig 2—Case 2 Ascites of eight months duration requiring thirty paracenteses. Immediate control of ascites with salt restriction. Prompt increase in urinary output and delayed rise in serum albumin.

38 per cent of normal, and esterase 13 units. Gamma globulin was elevated as indicated by a zinc turbidity of 32 units and a total serum globulin of 3.5 Gm per cent. Bromsulfalein retention was 40 per cent in 45 minutes. Ascitic fluid total protein was 1.0 Gm per cent, with albumin 0.7 Gm per cent.

On admission the patient was placed on a regular hospital diet and activity equivalent to that at home. On this regimen she continued to gain weight and ascites at her usual rate. The urinary output was small. Following a paracentesis which became necessary, she was put on a diet with NaCl restricted to 1 Gm per day. The response was immediate with a complete cessation of ascites accumulation and a rise in urinary output to about three times the previous output. This occurred despite a spontaneous decrease in fluid intake. A small amount of ascites, detectable by shifting dullness, persisted. This effect was sustained throughout her hospitalization and at home. After three months the patient was able to tolerate larger amounts of NaCl in her diet without forming ascites. She has remained well during the year since discharge and all ascites has disappeared.

A rise in serum albumin from a subnormal level of 2.4 Gm per cent to normal levels of 4 to 4.5 Gm per cent occurred.

Clinically there was marked improvement in this patient. As soon as she became adjusted to the diet she complained only of the flat taste of food without salt but this did not interfere with her eating a perfectly adequate diet. The termination of her need for paracenteses after having had thirty was a strong factor in raising her morale and insuring cooperation.

*Comment*—A low salt diet aided in restoring this patient to a fairly normal, active life, after having previously required almost weekly paracenteses for seven months. The urinary output likewise showed an immediate increase, while the serum albumin levels gradually rose to almost double the former levels within three weeks.

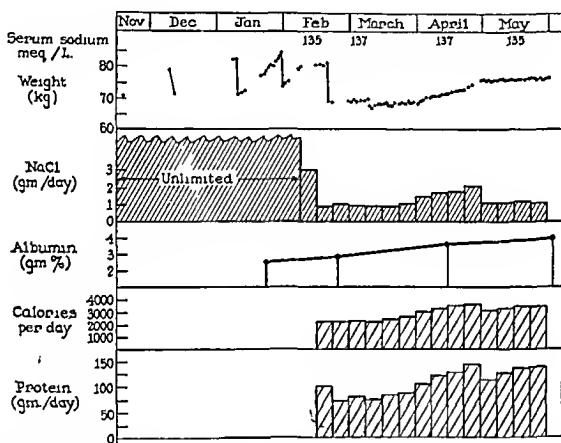


Fig 3—Case 3. Ascites formation controlled by NaCl restriction at 1 Gm per day. Reaccumulation of ascites on 1.5 to 2 Gm NaCl per day. Control of ascites again at 1 Gm per day. High protein intake (140 Gm per day) despite salt restriction to 1 Gm per day.

CASE 3—Fig 3 illustrates the course of a 54 year old wine drinker who on admission to the hospital was accumulating ascitic fluid at a regular rate. He had been hospitalized elsewhere on two occasions. Following an unexplained febrile episode he lapsed into hepatic coma for four days but gradually recovered. Three paracenteses were performed at the other hospital following the episode of coma. Since weights were available, these are illustrated in the weight curve of Fig 3. On physical examination the patient showed a small, hard liver and marked ascites with no edema. Following admission to the Rockefeller Hospital he was kept on a regular diet with normal sodium intake for three weeks. Ascitic fluid continued to accumulate and a fourth paracentesis was performed. Following the reduction of the NaCl content of his diet to 3 Gm per day there was an immediate slowing in the rate of ascites formation as is readily visible from the weight curve of Fig 3. The NaCl content was further reduced to approximately 1 Gm per day, and following a fifth paracentesis the patient ceased accumulating ascitic fluid for one month. The NaCl content of the diet was then gradually raised to 2 Gm per day. Ascitic fluid began to accumulate and the weight curve illustrated in Fig 3 can be seen to rise with progressive increments.

of salt intake. When NaCl was reduced again to 1 Gm per day, ascitic fluid again ceased accumulating and the weight curve flattened out. By means of protein supplements, as much as 140 Gm of protein were supplied a day during this latter period despite the very low NaCl intake.

Improvement on the low salt regimen was very striking. The discontinuance of paracenteses caused the patient to feel better and to eat better and resulted in a rise in the albumin concentration in the serum. The limitation of salt was very well tolerated. After three months on the low sodium diet, the patient recovered sufficiently to tolerate a normal diet at home without ascites.

*Comment*—The degree of salt restriction necessary to control the ascites in this patient was well defined at about 13 Gm a day. Limitation below this level promptly resulted in a favorable therapeutic response with control of ascites and a marked increase in urine volume. By the use of protein and carbohydrate supplements it was possible to raise the patient's protein intake to 140 Gm per day and his caloric intake to over 3,000 calories despite limitation of sodium chloride to 1 Gm per day.

*CASE 4*—Fig. 4 illustrates the course of a 60 year old housewife who developed hepatic cirrhosis without known cause and had been steadily accumulating ascites for four years. She had had seventy-six paracenteses prior to admission, with removal of an average of 10 liters per tap followed by leakage of fluid from the wound for one to two weeks. After closure of the wound she consistently accumulated approximately 1 liter of ascitic fluid per day. Edema of the legs appeared as the intra abdominal pressure increased.

In the nine months prior to admission the patient had become progressively weaker. She had lost considerable body tissue and had had several episodes suggesting minor cerebrovascular accidents. In the course of the four years the patient had been hospitalized for all paracenteses, and the main therapeutic efforts were directed toward a highly nutritive diet with occasional trials of mercurial diuretics.

On admission she appeared chronically ill, with marked tissue wasting. The liver was slightly enlarged and hard. Laboratory findings were consistent with an advanced cirrhosis with a decrease in the proteins formed by the liver, serum albumin was 2.5 Gm per cent, prothrombin level 47 per cent of normal, fibrinogen 160 mg per cent, serum esterase 27 units. Bromsulfalein retention was 23 per cent. Ascitic fluid total protein was 1.1 Gm per cent, with albumin 0.5 Gm per cent.

On the hospital diet with unrestricted salt the patient continued to gain weight and ascites at her usual rate until two paracenteses were performed. Just before the second tap in the hospital, salt in the diet was limited to 1 Gm a day. The rate of accumulation of ascites was promptly slowed and eventually leveled off at a point just short of requiring a paracentesis. With the institution of the low salt diet, urine output which had been minimal (200 to 300 cc a day) except following mercurials approximately doubled. This occurred despite a voluntary reduction in fluid intake. Attempts to raise the fluid intake were futile, leading to nausea. This may have been due to impaired renal function presumably on an arteriosclerotic basis. Both prior to and during the period of sodium restriction the patient showed a constantly elevated nonprotein nitrogen, a urea clearance of 25 per cent, and urine specific gravity fixed at 1.010. The patient's diet averaged 60 Gm of protein and 2,000 calories daily, with NaCl limited to below 1 Gm a day.

Laboratory tests showed no change with the exception of a moderate rise in serum albumin levels, from 2.5 Gm per cent to 3.7 Gm per cent. During the entire course there were but insignificant variations in the hemoglobin levels. Clinically this patient improved for two months but then her dietary intake fell and she was returned to a normal diet. A second period on the low Na diet again proved of little permanent value and the patient died three months after returning to the normal Na intake.

*Comment*—The chronicity of this patient's illness with resultant debility and prolonged ascites plus the renal and cerebral arteriosclerotic complications provided a difficult test for this regimen. After seventy-eight paracenteses the rate of ascites formation was promptly slowed by salt restriction but the dietary intake was poor and the patient eventually died.

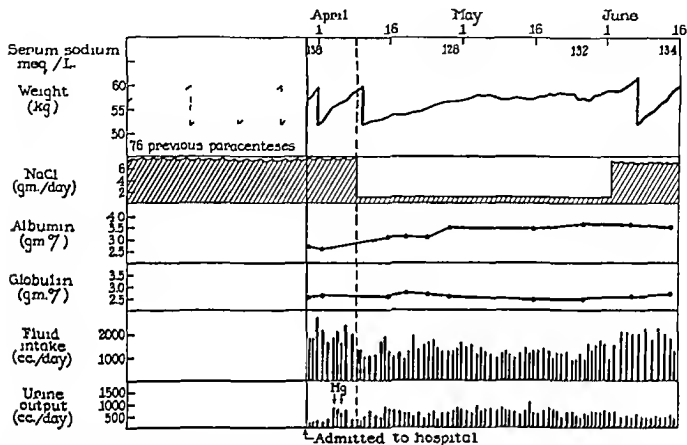


Fig. 4—Case 4. Ascites of four years duration requiring seventy-eight paracenteses. Effect of salt restriction in controlling formation of ascites. Increase in urine output and serum albumin level. Return of ascites and need for paracenteses on normal diet.

#### DISCUSSION

The results of rigid Na restriction have been described in detail in four of the thirteen patients studied. The remainder of the patients showed similar results with complete cessation of ascites formation when the NaCl was limited to 1 Gm. per day. Higher intakes caused ascites formation in direct proportion to the NaCl given. An average critical level of NaCl intake was obtained for these patients above which ascites formation occurred. This was 1.2 Gm. NaCl per day. The reason for this direct relation between NaCl intake and ascites was that the NaCl excretion in the urine was extremely low regardless of how much NaCl was furnished in the diet. The urinary NaCl excretion in eight of these patients on a diet of 7 to 9 Gm. NaCl per day ranged from 0.02 to 0.13 grams. Fecal NaCl excretion was higher, averaging 0.35 Gm. per day. With this low excretion of NaCl it is apparent that the large part of the NaCl intake is used to form ascitic fluid. Insensible NaCl loss was not measured but could be calculated from the NaCl balance data to be approximately 0.8 Gm. per day.

Excretion of water rose in all of the patients on the low sodium diet. When there was no Na supplied in the diet for ascitic fluid accumulation water was

exercised proportional to the intake. A high fluid intake had little effect in altering daily sodium excretion.

All but one of the thirteen patients were able to tolerate the low Na regimen for at least three months without ill effects. The one exception, Patient 4, showed a fall in fluid and calorie intake on the diet. Another patient developed muscle cramps after being on the diet for five months. These were relieved by supplying Na. The patient had done well on the diet without forming ascites prior to the onset of the cramps. Serum Na levels were followed in all of the patients. A fall in serum Na of approximately 3 meq per liter usually occurred. One patient who received mercurials in addition to the low Na diet showed the greatest fall, 12 meq per liter.

Nitrogen balance studies were carried out in two of the patients. These patients were in negative balance prior to Na restriction because of the loss of ascitic nitrogen. When ascites formation ceased on the low Na diet, the patients reverted to positive nitrogen balance.

All of the patients demonstrated a rise in serum proteins on the low Na diet. Fig. 5 shows a group of determinations on the serum and ascitic fluid of one of the patients (Case 1). The rise in albumin and in colloid osmotic pressure was approximately equal in the two fluid compartments, demonstrating the equilibrium which exists. The total protein of serum and ascitic fluid rose considerably more than the albumin. Plasma volume determinations showed little change and thus did not account for the rise. Some of the other patients demonstrated a 10 per cent fall in plasma volume accounting for a portion of the protein rise. The patients who responded best to the diet and gained body weight soon after treatment was started showed a rise in serum albumin that was sometimes greater than the total protein rise. This was due to a fall in globulins. The selective rise in albumin was a sign of improvement in liver protein synthesis. This did not occur in the chart shown (Fig. 5) nor in most of the other patients. The rise in these patients of all the protein components appeared to be the result of cessation of protein loss in the ascitic fluid permitting the proteins to accumulate in the body.

The use of newly available low Na protein supplements permitted the administration of as much as 150 Gm of protein per day on the low Na diet. The patients showed considerable individual variation in their ability to take different supplements, but of those which were used there usually was one which was readily tolerated by each patient. Improvement appeared to be more rapid when the protein was kept above 100 grams. However, some of the patients responded well at a level of 70 Gm of protein per day.

The general clinical results of a three-month period on the low NaCl diet were evaluated by studying the effect of normal Na intake on ascites at the end of the three months. Four patients recovered sufficiently during this time to enable them to excrete the increased Na and they did not reform ascites. These patients gradually absorbed the remaining ascitic fluid and returned to a normal life. Eight patients were still unable to excrete larger amounts of Na and accumulated ascites on the normal diet. These patients had not progressed during the three months of Na restriction and all of them appeared

slightly improved but they had not been cured. Na restriction was reinstituted in these individuals. Three of these have since showed increased Na excretion representing loss of ascitic fluid after more than six months of therapy. Others in the group are still being treated with combined albumin, liver extract, and low Na therapy. One of these patients died of esophageal hemorrhages. Patient 4 who did not tolerate the low Na diet also died.

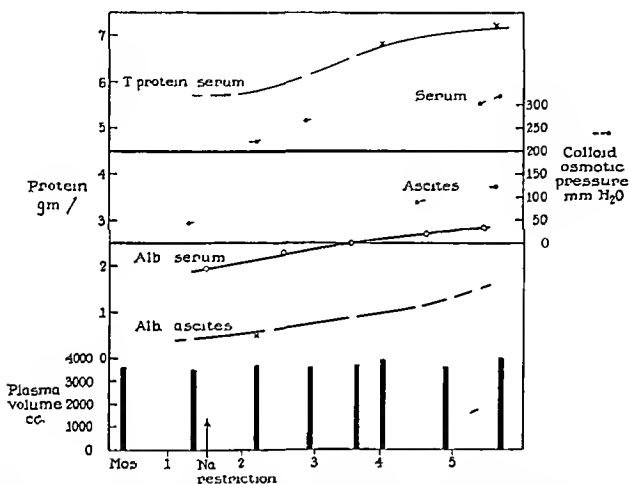


Fig 5—Rise in values for determinations of proteins and colloid osmotic pressure in serum and ascitic fluid following Na restriction.

In all of the patients the diet appeared to be beneficial in stopping the malignant course of events brought about by removal of protein through paracenteses in the already depleted individual thus enabling dietary therapy to become effective. The long term clinical results are difficult to evaluate but the fact that ascites formation could be completely controlled by rigid Na restriction was clearly evident.

#### SUMMARY

1 The results of rigid NaCl limitation in the diet of thirteen patients with cirrhosis of the liver and long standing ascites are presented.

2 Ascites formation ceased for three months in twelve of the thirteen patients. Urine output increased in each case commensurate with decreased fluid retention.

3 Four patients did not reform ascites when a normal NaCl intake was tried after three months; eight patients did reform ascites.

4 Serum protein levels showed a rise following therapy. This is explained primarily on the basis of retention in the serum of protein which previously had been lost in the ascitic fluid.

5 A high protein and high caloric diet could be maintained despite rigid salt limitation.

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# THE HYPERBILIRUBINEMIC EFFECT OF SODIUM NICOTINATE

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## INTRODUCTION

NEW techniques are often instrumental in permitting important developments in the understanding of biological and clinical problems. In 1941, Villa<sup>13</sup> claimed that treatment with nicotinamide produced a striking remission of jaundice in different types of hepatic dysfunction provided the liver damage had not reached the irreversible stage. Apparently while investigating the mechanism of this therapeutic effect Matter<sup>9</sup> observed that the injection of 30 mg of sodium nicotinate intravenously in normal individuals was followed by a sharp rise of the level of indirect reacting bilirubin in serum. This technique has permitted interesting observations on some aspects of the metabolism of biliary pigments in normal and pathologic conditions.<sup>8</sup> The results obtained so far in more than twenty normal subjects and seventy eight patients with various disorders of the liver will now be reported.

## THE TECHNIQUE OF THE TEST

Ten cubic centimeters of blood are collected from the patient who has been fasting for the previous twelve hours and 30 mg of sodium nicotinate dissolved in 10 cc of saline solution are slowly injected through the same needle. The injection is followed almost immediately by a sensation of flushing and intense heat. Temporary marked vasodilatation is also observed particularly in the face and extremities. Ten cubic centimeters of blood are again collected 90, 210, and 480 minutes after the injection. The intervals have been selected because they best show the changes of serum bilirubin level during the test, both in normal and pathologic conditions. Patients may be given a light meal after the third sample of blood has been collected to avoid an excessively long period of fasting. This practice does not influence the result of the test.

The tubes containing the blood are left in the oven at 37° C for one hour and the serum is then separated by centrifugation. To prevent hemolysis (which represents one of the major causes of error in the determination of the bilirubin level) the blood must be obtained with a perfectly dry syringe and needle† by a clean venipuncture and without forcing. Total and direct reacting bilirubin are determined in each sample with the method of Jendrassik and Grof<sup>6</sup> and the value of bilirubin of the indirect reacting type is calculated by difference.

## RESULTS

*Effect of the Intravenous Injection of Sodium Nicotinate on Bilirubinemia*—Following the intravenous injection of sodium nicotinate in normal subjects the total bilirubin level of serum rises sharply, reaches a maximum value after ninety minutes and then decreases slowly, returning to normal

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†Coating of syringes and needles with the film Ilkone (General Electric Dri-Film 9987) permits one to obtain serum free of hemolysis almost constantly. Technical details are given in a previous paper.

figures within six to eight hours. Ordinarily the rise in circulating bilirubin is due to the "indirect reacting type" since no bilirubin of the direct reacting type appears in circulation. If the bilirubin variations are followed at regular

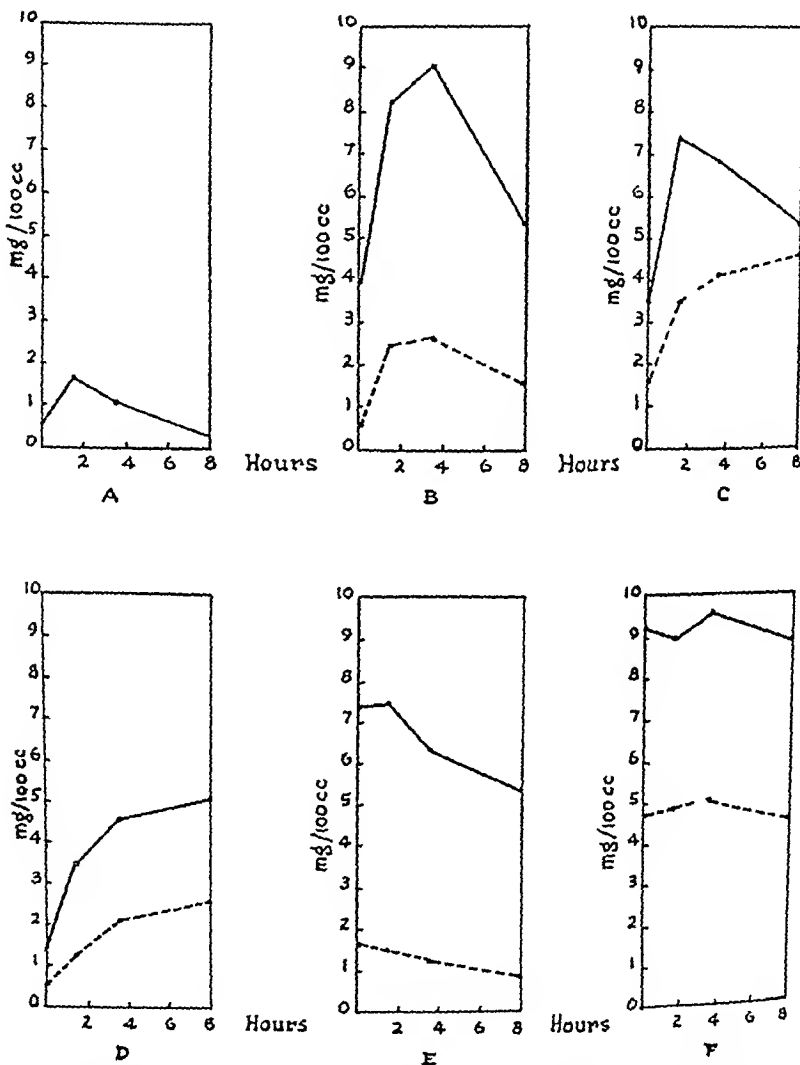


Fig 1—Different types of hyperbilirubinemic curves obtained in normal subjects and in patients with various liver disorders following the intravenous injection of 30 mg of sodium nicotinate

(The continuous lines represent the level of total serum bilirubin the broken ones the level of serum direct reacting bilirubin)

intervals and plotted against time, a curve is obtained with a sharp, short ascending and a prolonged, slowly descending segment (Fig 1, A shows an average normal curve, derived from the analytic values obtained in twenty-two normal subjects). Serum cholesterol and bile salts are also influenced by the intravenous injection of sodium nicotinate (Table III), but so inconsistently that their variations do not present any clear significance. This phenomenon appears to be specific for sodium nicotinate. Chemically related substances

TABLE I LEVELS OF BILIRUBIN CHOLESTEROL BILE SALTS, CHLORIDES AND URIC ACID OF SERUM IN A PATIENT WITH CIRRHOSIS OF THE LIVER FOLLOWING THE INTRAVENOUS INJECTION OF SODIUM NICOTINATE (30 MG)

TIME AFTER INJECTION	0 MIN	90 MIN	120 MIN	180 MIN
Total bilirubin (mg per 100 ml)	3.99	8.12	9.02	5.65
Direct reacting bilirubin (mg per 100 ml)	0.33	1.05	1.07	0.89
Cholesterol (mg per 100 ml)	85	130	78	71
Bile salts (mg per 100 ml)†	0	20	8	0
Chlorides (Gm per 100 ml)	7.37	6.00	7.02	7.11
Uric acid (mg per 100 ml)	4.88	4.35	4.16	4.21

\*Determined with the method of Autenrieth

†Determined with the method of Cottet

such as nicotinamide and nikethamide and drugs sharing with sodium nicotinate some pharmacologic effects (histamine epinephrine and acetylcholine) either cause no modification of the serum bilirubin level or do so to a much lesser extent than sodium nicotinate (Table II). No significant changes of red blood cells and reticulocyte count (Table III) or of the blood level of chlorides and uric acid (Table I) follow the intravenous injection of sodium nicotinate.

TABLE II EFFECT ON THE SERUM BILIRUBIN LEVEL OF THE PARENTERAL ADMINISTRATION TO NORMAL SUBJECTS OF SUBSTANCES CHEMICALLY OR PHARMACOLOGICALLY RELATED TO SODIUM NICOTINATE

SUBSTANCE	DOSE AND ROUTE OF ADMINISTRATION	SERUM BILIRUBIN LEVEL (MG %)			
		TIME AFTER INJECTION (MIN)			
		0	30	90	180
Normal control		0.36		0.32	0.43
Sodium nicotinate	30 mg intravenously	0.34	0.58	1.01	0.74
Histamine, hydrochloride	1 mg, intramuscularly	0.50	0.61	0.55	0.58
Epinephrine, hydrochloride	1 mg intramuscularly	0.70	0.86	0.67	0.50
Acetylcholine, hydrochloride	100 mg intramuscularly	0.48	0.43	0.51	0.50
Nikethamide	850 mg intramuscularly	0.40	0.42	0.40	0.38
Nicotinamide	100 mg, intravenously	0.58		0.61	0.60

TABLE III TOTAL SERUM BILIRUBIN LEVEL, RED BLOOD CELL AND RETICULOCYTE COUNT IN A NORMAL SUBJECT FOLLOWING THE INTRAVENOUS INJECTION OF 30 MG OF SODIUM NICOTINATE

TIME AFTER INJECTION	0 MIN	90 MIN	120 MIN	180 MIN	24 HR.	48 HR.	72 HR.
Total serum bilirubin (mg per 100 ml)	0.75	1.40	1.05	0.89	-	-	-
Red blood cells (millions)	4.86	5.00	4.60	4.91	4.87	4.92	4.85
Reticulocytes (per thousand)	5	5	8	5	8	13	9

*The Chologogic and Choleretic Effect of Sodium Nicotinate*—When 30 mg of sodium nicotinate are given intravenously to normal subjects in whom a Reluss tube has been passed previously into the duodenum a copious flow of bile begins about ten minutes after the injection. For the first five minutes

the bile collected has the characteristics of bile A, then, for about twenty minutes, of bile B, and finally of bile C. The total amount of bile which is collected in one hour's time is 200 to 250 cc, far greater than the amount obtainable through ordinary stimulation with the Meltzer-Lyon technique. In the serum bilirubin level is determined simultaneously, it appears to be only minimally increased. The same low type of hyperbilirubinemic curve is obtained when the biliary flow is stimulated with intraduodenal introduction of  $MgSO_4$ , thirty to sixty minutes before the intravenous injection of sodium nicotinate, and the bile obtained is, at least in part, extracted through the Rehfuess tube.

The first two findings suggest a direct stimulation of the bile excreting function of the liver by sodium nicotinate. This has been, moreover, proved directly from the study of a patient suffering from chronic hepatocholangitis in which a surgical biliary fistula had been therapeutically established. This case, which offered a rare opportunity of following the modifications of the bilirubin level of bile as compared with those of serum bilirubin during the performance of the sodium nicotinate test, has been presented in detail elsewhere.<sup>7</sup> Notwithstanding the presence of the fistula, the concentration of stercobilin (determined in the feces with the method of Watson<sup>15</sup>) showed a considerable amount of bilirubin still flowing into the intestine. The injection of sodium nicotinate in this patient was followed by a limited rise in the serum bilirubin level. Its consequent decrease toward the initial value was accompanied in direct time relationship by an increase of bilirubin concentration in bile.

*The Effect of the Intravenous Injection of Sodium Nicotinate on Urobilinuria*—The cholagogic and choleretic action of sodium nicotinate provides larger amounts of urobilinogen for reabsorption from the intestinal tract. This effect is further enhanced by the vasodilatation caused by the substance, particularly pronounced in the splanchnic area. A corresponding rise in the amount of urobilin eliminated could, therefore, be expected.

Studies on the changes in elimination of urobilin after the intravenous injection of sodium nicotinate have been carried out in twelve normal subjects and in a few patients with various grades of liver dysfunction. Urobilin level in urine has been determined with the original method of Heilmeyer and Krebs.<sup>5</sup> It has been found consistently that, following the intravenous injection of sodium nicotinate, the urinary concentration of urobilin rises to from five to eight times the original values, reaches a maximum value two to three hours after the injection, and then slowly returns to the normal level in twenty-four hours (Fig 2, Table IV). This rise in concentration of urobilin is roughly proportional to that of the indirect reacting bilirubin of serum, although somewhat delayed. It is greatly pronounced (ten to fifteen times the original values) in patients presenting serious liver dysfunction.

In patients in whom direct reacting bilirubin appears or increases in excretion as an effect of the injection of sodium nicotinate, a contemporary appearance of bilirubin or rise in its concentration is observed in the urine.

TABLE IV. MODIFICATIONS OF THE LEVEL OF TOTAL AND DIRECT REACTING BILIRUBIN OF SERUM AND OF UROBILIN FOLLOWING THE INTRAVENOUS INJECTION OF 30 MG OF SODIUM NICOTINATE

TIME AFTER INJECTION	0 MIN	90 MIN	110 MIN	180 MIN	24 HR.
<i>(a) Subject M S ♂ age 29 normal</i>					
Total bilirubin (mg per 100 ml)	0.22	0.97	0.68	0.19	—
Direct reacting bilirubin (mg per 100 ml)	0	0.18	0	0	—
Urobilin (mg per 100 ml)	0.042	—	0.526	0.372	0.058
<i>(b) Patient N H age 48 ♂ suffering from chronic hepatocholangitis</i>					
Total bilirubin (mg per 100 ml)	3.46	6.02	5.48	4.89	—
Direct reacting bilirubin (mg per 100 ml)	1.17	1.83	1.97	1.64	—
Urobilin (mg per 100 ml)	0.094	0.465	1.037	0.702	0.172

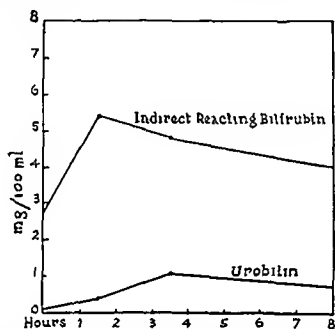


Fig. 2.—The effect of the intravenous injection of 30 mg of sodium nicotinate on the level of serum indirect reacting bilirubin and the level of urobilinuria in a patient with chronic hepatocholangitis. (The values of indirect reacting bilirubin and of urobilin are expressed in milligrams per 100 ml of serum and urine respectively.)

*Hyperbilirubinemic Curves in Normal and Pathologic Conditions*—As described previously, the intravenous injection of sodium nicotinate in normal subjects is followed by a rise in the level of indirect reacting bilirubin of serum, which returns to normal within eight hours.

The liver cell very probably disposes of the sudden load of indirect reacting bilirubin caused by the sodium nicotinate test by transforming it to direct reacting bilirubin and certainly eliminates the latter. A curve of the type shown in Fig. 1, A is therefore obtained when both bile forming\* and bile excreting functions of the liver are normal. The curve is usually found (a) in normal subjects, (b) in patients with moderately abnormal liver function but no clinical jaundice, (c) in a few patients presenting mild clinical jaundice and a serum bilirubin level slightly higher than normal. Some of the patients of the third group may also present a small amount of circulating direct bilirubin which is only insignificantly modified during the course of the test and always returns to the initial value at the end of the eight hour period. Often, at the end of the

\*To be understood as the capacity probably possessed by the liver cell of transforming the indirect to direct reacting bilirubin of elimination and indicated by the gradual decrease of the circulating bilirubin of the indirect reacting type.

the bile collected has the characteristics of bile A, then, for about twenty minutes, of bile B, and finally of bile C. The total amount of bile which is collected in one hour's time is 200 to 250 c.c., far greater than the amount obtainable through ordinary stimulation with the Meltzer-Lyon technique. If the serum bilirubin level is determined simultaneously, it appears to be only minimally increased. The same low type of hyperbilirubinemic curve is obtained when the biliary flow is stimulated with intraduodenal introduction of  $MgSO_4$ , thirty to sixty minutes before the intravenous injection of sodium nicotinate, and the bile obtained is, at least in part, extracted through the Rehfuess tube.

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In patients in whom direct reacting bilirubin appears or increases in circulation as an effect of the injection of sodium nicotinate, a contemporaneous appearance of bilirubin or rise in its concentration is observed in the urine.

No tissue deposits of bile pigments are known to exist, at least in normal conditions,<sup>1</sup> nor is the injection of sodium nicotinate followed by changes in level of blood chlorides and ure acid (substances which are easily mobilized from tissues). Another possibility namely that the hyperbilirubinemia may be due to an exaggeration of normal blood destruction, has no clear cut experimental evidence in its favor. No changes of red blood cell and reticulocyte count follow the intravenous injection of sodium nicotinate although these changes might be very small yet significant (more important they could be masked by variations of hemoconcentration). However hyperbilirubinemic curves of normal type are usually obtained in patients with increased fragility of erythrocytes.\*

Our findings suggest that the increase of indirect reacting bilirubin of serum is due to the increased reabsorption from the intestine of a biliary pigment (possibly mesobilirubin). This tentative conclusion is based on strong but, so far, indirect evidence (a) the powerful chologogic and choleretic effect of sodium nicotinate and (b) its pronounced vasodilatory action on the vessels of the splanchnic area. As a result the reabsorption from the intestinal tract of components of bile is greatly increased. (Exaggerated reabsorption of a chromogenous bilirubin derivative—urobilinogen—following the intravenous injection of sodium nicotinate is shown by the corresponding changes in elimination of urobilin.) Further evidence shows that availability of bile in the intestine and hyperbilirubinemic effect of sodium nicotinate are directly related as proved by two main findings (a) the hyperbilirubinemic effect of sodium nicotinate is limited or absent in normal subjects when biliary flow is first stimulated by means of  $MgSO_4$  and the secreted bile extracted as completely as possible by means of a Rehfuess tube passed into the duodenum (b) patients with complete biliary obstruction do not present any hyperbilirubinemic response to sodium nicotinate.

The conclusion suggested is not supported by the accepted theories on the metabolism of biliary pigments. However the Italian school of Zoja<sup>16</sup> has strongly claimed that serum bilirubin of the indirect reacting type represents reabsorbed mesobilirubin which has escaped the hepatic filter through lymph channels or the porta-caval anastomoses. Notwithstanding the demonstration by Colangiuli<sup>3</sup> that mesobilirubin and indirect reacting bilirubin behave in a similar manner with various chemical reagents the theory still awaits more conclusive evidence and is not generally accepted. It would however offer an explanation of our findings since the increased flow of bile and the vasodilatation induced by sodium nicotinate could exaggerate the reabsorption of mesobilirubin† and consequently raise the serum level of indirect reacting bilirubin.

The increased elimination of urobilin which follows the intravenous injection of sodium nicotinate is another finding of clinical interest and practical

\*A very low hyperbilirubinemic curve was observed in a patient with Werlhof's disease recently splenectomized. This finding is difficult to interpret and thus does not specifically support or deny the view that exaggerated blood destruction is the cause of the hyperbilirubinemia due to sodium nicotinate.

†No hyperbilirubinemia follows the intravenous injection of nicotineamide which is only slightly less active than sodium nicotinate in increasing the biliary flow but has not possessed any vasodilatory effect. This indirect evidence would suggest that the hyperbilirubinemia depends more on the vasodilatory action than on the chologogic and choleretic effect of sodium nicotinate.

importance. The effect is probably due to a double mechanism: (a) the presence in the intestinal tract of a large amount of urobilinogen available for reabsorption, due to the cholagogic and choleretic effect of sodium nicotinate, (b) the vasodilatory effect of the substance on the splanchnic vascular area, which increases the possibility of the chromogen being adsorbed directly into the general circulation through portacaval anastomotic vessels. The coordination of these two mechanisms explains the magnitude of the increased elimination of urobilin in the urine after intravenous injection of sodium nicotinate. From a clinical viewpoint the observation shows that *large quantities of urobilin may appear in the urine even when no liver damage or hyperhemolysis can be demonstrated*. This last finding indirectly confirms earlier experiments of Mell<sup>10</sup> who was able to show in dogs that the rise of pressure within the portal system (determined with experimental occlusion or direct compression of the portal vein, or pneumothorax of the right side) causes increased elimination of urobilin. When the pressure within the portal tree is increased, an amount of urobilinogen greater than normal reaches the general circulation and is finally eliminated as urobilin. A similar mechanism can be cited to explain the pronounced urobilinuria of clinical conditions (pneumothorax of the right side, pylethrombosis, early cirrhosis of the liver) in which liver dysfunction is absent or too limited to account for the finding. It could also explain the daily fluctuations and the postprandial increase of physiologic urobilinuria.

The findings presented in this paper might have possible diagnostic and therapeutic applications. That the sodium nicotinate technique could be used as a test of liver function is obvious. The technique, producing a prompt and conspicuous rise of the serum indirect reacting bilirubin, tests the ability of the hepatic cell to dispose of a sudden and large load of bilirubin and evaluates both the bile-forming and the bile-excreting functions of the liver. Compared with the tolerance tests of exogenous bilirubin or with other techniques in which a rise of the serum bilirubin level is determined indirectly by the injection of different substances (epinephrine, biliary salts, desoxycorticosterone acetate) or other methods (ingestion of fats and carbohydrates, diathermy and roentgen irradiation of the hepatic and splenic areas), the sodium nicotinate test has the advantage of causing a prolonged and conspicuous hyperbilirubinemia with a technique both simple and safe. Unfortunately, studies on seventy-eight patients presenting various liver disorders show the pattern of the hyperbilirubinemic curve after intravenous injection of sodium nicotinate to present no definite correlation with the anatomic or clinical diagnosis of the case, the total hepatic function evaluated with a "profile" of liver function tests, or the results of any other individual test. This finding is not attributable to an intrinsic defect of the sodium nicotinate technique, but to the fact that bilirubin excretion is widely dissociated from other functions of the liver. It may remain normal even in cases of serious cellular damage, or be involved early in rather benign conditions. The results of the test, therefore, cannot be used as an indication of the total function of the liver nor considered as a means of differential diagnosis between obstructive and hepatocellular jaundice, since a dia-



nostically typical curve (first type) is obtained only in cases of complete obstruction of the biliary tract. The sodium nicotinate technique remains mainly a test of the bilirubin excreting function of the liver. Under certain conditions, it may also be useful in the prognostic evaluation of jaundice. For example in cases of infectious hepatitis still with evident jaundice the test may give a normal result, indicating the restoration of a normal excretion of bilirubin and forecasting the regression of the icterus at an early date. In other cases jaundice may be due rather to sluggishness than insufficiency of the bilirubin excreting function as in icteric malaria. The sodium nicotinate curve will then show a normal pattern.

Sufficient evidence has been given to show that sodium nicotinate when administered intravenously exercises a powerful cholagogic and choleric action. This effect has been proved by means of duodenal intubation in normal subjects and in a patient with surgical biliary fistula. Experiments utilizing the same techniques have also shown that intravenous nicotinamide or sodium nicotinate or nicotinamide administered by mouth in therapeutic doses also possess the same action although slightly less pronounced. Although these findings suggest a possible therapeutic use of both substances in jaundice which has not yet reached the irreversible stage, much more additional evidence is required before these conclusions can be given practical clinical application.

This communication should be considered of a preliminary character and the results presented here certainly do not exhaust the possibilities of experimental and clinical investigation offered by the sodium nicotinate test. It is unfortunate that the related observations cannot be completed and integrated with animal experimentation because no hyperbilirubinemia can be determined in dogs and rabbits with the technique. However, further work in human beings is contemplated, employing this test, to obtain more information on the normal and pathologic metabolism of biliary pigments.

#### SUMMARY AND CONCLUSIONS

1 The intravenous injection of 30 mg. of sodium nicotinate in normal subjects is followed by (a) a rise of the serum level of indirect reacting bilirubin, which reaches a maximum after about ninety minutes, then slowly returns to the initial value within six to eight hours, (b) almost immediate stimulation of the secretion and excretion of bile (bilirubin), (c) increased elimination of urobilin which reaches a maximum two to three hours after the injection and returns to normal values in about twenty four hours.

2 In pathologic conditions two main modifications of the hyperbilirubinemic effect of sodium nicotinate are observed (a) a failure of the bilirubin level to return to normal values within the experimental period of eight hours (b) the appearance in circulation of bilirubin of the direct reacting type. Their various combinations characterize different types of curves obtained in patients with liver disorders. These curves are not consistently related to the anatomic or clinical condition of the patient. Therefore the hyperbilirubinemic effect cannot be utilized as a test of liver function or for the differentiation between hepatogenous and obstructive jaundice.

3 The interpretation of the ascending part of the hyperbilirubinemic curve is complex, experimental evidence suggests that the rise in level of the indirect reacting bilirubin in serum might be due to increased reabsorption of meso bilirubin from the intestine. The descending part is in direct relationship to the bile-excreting function of the liver.

4 The increased elimination of urobilin is very likely due to the increased reabsorption of urobilinogen, because of the vasodilatory, chologogic and choleretic effects of sodium nicotinate. The finding proves that large quantities of the chromogen may appear in the urine even when no liver damage or hyperhemolysis can be demonstrated.

5 Both sodium nicotinate and nicotinamide, when given intravenously or orally, exert a powerful choleretic and chologogic effect.

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# CHOLESTEROL DESOXYCHOLIC ACID A STABLE ANTIGEN FOR USE IN A FLOCCULATION TEST FOR LIVER DYSFUNCTION

## I COMPARISON WITH THE HANGER CEPHALIN CHOLESTEROL TEST

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SINCE the introduction of the Hanger test<sup>1,2</sup> it has found many proponents.<sup>3,4</sup> I had an opportunity to employ this test as well as other liver function studies in a large series of cases in military personnel.

There were a few pitfalls associated with the cephalin cholesterol flocculation test as it was first performed by me. Bacterial contamination was found to give rise to false positives especially in an overheated room. Furthermore the reaction was photosensitive.<sup>11</sup> The latter was reported also by Neefe and Remhold.<sup>12</sup> To avoid bacterial contamination sulfanilamide was dissolved in the 0.85 per cent saline in a concentration of 1:1,000 and later a 1:10,000 solution of Merthiolate was employed. With both of these agents there was found no interference with the sensitivity of the reaction. To avoid the false positives observed with exposure to light especially direct sunlight the tubes were kept in a dark cupboard.

It was observed early that there was a considerable disparity of results obtained in the Hanger tests when parallel tests were set up with two commercial antigens, Wilson and Difco. The former seemed to be much less sensitive. It appeared likely that such variation between the two antigens may have been due to differences in the cephalin. Previously Mateer and co-workers had indicated that the state of the cephalin affected the sensitivity of the test.<sup>6,14</sup> Later Lippincott and associates<sup>1</sup> reported a discrepancy of results obtained in patients with vivax malaria employing the Difco and Wilson antigens. These authors also found the Wilson antigen to be less sensitive.

Kabat, Hanger, Moore and Landow<sup>16</sup> showed that false positive tests were obtained with known negative sera when the sera was inactivated for thirty minutes at 56° C. This observation also was made by me in using similar heat inactivated sera.

There is no attempt made in this paper to detract from the value of the Hanger reaction as a liver function test in fact adequate confirmation of the reliability of the test is presented herein. This investigation was undertaken because of the following: (1) the instability of the cephalin in the preparation of the antigen; (2) the difficulty in preparing the antigen or the expense in purchasing commercial preparations of the antigen; and (3) the lack of availability

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of the antigen or the components for its preparation in overseas hospital installations. An attempt was therefore made to prepare a similar antigen from readily available compounds, but one that would be stable. Cephalin is an unstable phospholipid, which readily undergoes oxidation. It appeared likely that the chief function of this substance in the preparation of the Hanger antigen was to render the antigen more soluble in the aqueous media employed in the flocculation test. This fact was also suggested by MacLagan,<sup>1</sup> who, during the development of the thymol turbidity test, suggested that the turbidity obtained with the sera of patients manifesting parenchymatous liver disease was due primarily to phenol and its substitution products. He showed a definite relationship between the molecular weight, solubility, and the precipitating power. He indicated that higher homologues such as cholesterol failed to give positive results because of their lack of solubility.

Consequently, I sought a stable substance of known chemical composition that would increase the solubility of cholesterol. Veizán and Kúthy<sup>18, 19</sup> demonstrated that free bile acids have the ability to keep fatty acids and other acholic components in aqueous solution. This appeared to be accomplished by the formation of coordination compounds and was confined to the alkaline side of the neutral point. These authors also showed that lecithin and other substances exert an influence of their own on solubility in a mixture and may supplement the effect of the bile acids. The enhanced solubility of cholesterol in bile and bile acids solutions was mentioned as early as 1868 by Kuchne.<sup>20</sup> Finally, diffusion experiments by Schoenheimer and Hidina<sup>21</sup> showed that cholesterol was more strongly associated with desoxycholic acid than any other bile acid.

Hence, desoxycholic acid was selected as the compound of choice, to replace cephalin in the Hanger antigen. Desoxycholic acid has been used in the preparation of a variety of coordination compounds, including the phenanthrene hydrocarbons. It has the property of forming loose compounds with steroids, thereby broadening the solubility range of the combined substance. Initially a coordination compound of cholesterol-desoxycholate was prepared by fusing one molecular weight each of cholesterol and desoxycholic acid with the aid of heat. A cholesterol-desoxycholate was also prepared by the method of Downie, Stent, and White<sup>22</sup> by crystallization from petroleum ether. Subsequently a simpler preparation was devised, which eliminated the necessity for the formation of this coordination compound entirely.

#### METHOD

*Preparation of the Stock Antigen*—1.2 Gm of chemically pure desoxycholic acid were dissolved in 16 cc of absolute alcohol with the aid of gentle heating, then 0.6 Gm of chemically pure cholesterol was dissolved in this solution of desoxycholic acid. This yields an alcoholic solution of cholesterol which was of the same concentration as that of Hanger. This stock antigen was stored in a tightly stoppered bottle in the refrigerator.

*Preparation of the Cholesterol Desoxycholic Acid Emulsion*—The working antigen was prepared by placing 30 cc of N/60 NaOH in a small beaker and heating to about 70° C. The flame was removed and 1 cc of stock antigen was added, a drop at a time, with constant stirring. The mixture was then slowly brought just to the boiling point with constant stirring. The resultant emulsion was then allowed to cool to room temperature and transferred to two 15 cc centrifuge tubes. These were centrifuged at 1,500 rpm for five

minutes to remove any coar o particles. The supernatant milky emulsion was then poured off and stored in a stoppered test tube. This emulsion keeps for days in the refrigerator.

*Procedure*—To 4 cc of phosphate buffer of pH 7.8 in a small test tube or 15 cc centrifuge tube such as Hanger employed, was added 0.2 cc of serum. The contents were then mixed by inverting several times. Then 1 cc of antigen emulsion was added and the contents were vigorously shaken, stoppered and allowed to stand. The tubes were examined at twelve, twenty four and forty eight hours for flocculation and were reported as negative, +, ++, +++, +++++, as in the Hanger test. Two control tubes were prepared at the same time. One contained known negative serum and the other contained just the buffer and emulsion in the same proportions as in the test. Flocculation of ++ or more in forty eight hours was considered positive.

Parallel Hanger tests were performed in all sera tested. Simultaneous thymol turbidity, icterus index, serum bilirubin albumin/globulin ratio, cholesterol, total and esters, alkaline phosphatase and, in many instances bromsulphalein, heterophile antibody, and urine urobilinogen were also carried out. These latter will be reported in a subsequent paper.<sup>26</sup>

There were 600 tests performed on 485 individuals. The type of cases utilized are depicted in Table I.

TABLE I CASES STUDIED

DIAGNOSIS	NUMBER OF PATIENTS	NUMBER OF TESTS
Infectious hepatitis	143	174
Obstructive jaundice	14	22
Cirrhosis of liver	5	12
Cholecystitis	8	8
Infectious mononucleosis	38	66
Vivax malaria	82	116
Miscellaneous	28	34
Normal	168	168
Total	485	600

Where possible the sera of these patients were tested during the various phases of the disease, i. e., at the onset during subsidence during recrudescence, and following clinical recovery. The diagnoses in the various cases were based on appropriate laboratory studies and clinical evaluation.\*

## RESULTS

The results obtained in the individual cases will be published subsequently together with the other laboratory data mentioned previously. A summary of the data is herein presented.

The results obtained in the sera of patients in various stages of infectious hepatitis are shown in Table II.

TABLE II FLOCCULATION TESTS IN CASES OF INFECTIOUS HEPATITIS

NUMBER OF CASES	NUMBER OF TESTS	% HANGER POS	% CDF* POS	CLINICAL STAGE
50	76	96	97	Active (acute)
64	73	92	95	Progressive
—	25	48	11	Recovered†
144	174	78.6	67.6	

CDF = cholesterol desoxycholic acid flocculation test.

† Icterus index serum bilirubin normal.

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Physically, the flocculant of the cholesterol desoxycholeic acid flocculation test appears to be finer and to pack more firmly. An effort will be made to perform these tests subsequently.

The evidence reported herein would suggest that the Hanger and cholesterol desoxycholeic acid flocculation tests could be employed interchangeably in the diagnosis and follow up of cases of hepatocellular diseases.

#### SUMMARY

1. A new antigen is described in which desoxycholeic acid was substituted for cephalin in the cephalin cholesterol flocculation test.

2. There were 600 tests on 475 patients carried out employing the cholesterol desoxycholeic acid (CDI) and the cephalin cholesterol flocculation test with 94 per cent agreement.

3. The cholesterol desoxycholeic acid flocculation test compared closely with the Hanger test in specificity and sensitivity. The mechanism of action in the two tests appeared to be closely related.

4. The cholesterol desoxycholeic acid flocculation test appeared to be of advantage in regard to stability, ease of preparation and economy.

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## A CRYOGLOBULIN PRESENT IN HIGH CONCENTRATION IN THE PLASMA OF A CASE OF MULTIPLE MYELOMA

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THE first reported case of a reversible cold precipitation of plasma protein is that of Wintrobe and Buhl<sup>1</sup> (1933). This and other cases that have been reported since that time are summarized by Lerner and Watson (1947), who give the relevant bibliographies. In their paper a new case is described which is particularly valuable because of the completeness of the studies. An extensive physical chemical study of the protein is described in a separate paper by Lerner and Greenberg.<sup>2</sup> All of these cases gave immediate gross precipitation of protein on cooling the plasma and resolution of the precipitate on warming. Because of this property Lerner and Watson have suggested the name "cryoglobulin" for this class of proteins.

Since the publication of the paper of Lerner and Watson, a case with Raynaud's syndrome having a protein of this class in the plasma was reported by Hansen and Faber<sup>3</sup> (1947). Olhagen<sup>4</sup> (1948) has also described three cases that merit special mention. All three gave spontaneous crystalline precipitates, although the crystal structure was not the same in any two cases. In Case I (diagnosis myeloma, total plasma protein 10.1 Gm per cent) the content of spontaneously precipitating protein was 1.4 Gm per cent of plasma. In spite of this high concentration it did not precipitate immediately on cooling, and after warming to 37° C. redissolved slowly (twenty to thirty minutes). There is a question whether Olhagen's Case II (diagnosis, myeloma, total protein 13.0 Gm per cent) should be listed as a cryoglobulin because the precipitation was "on the whole independent of the outer temperature but slightly faster in the cold." The spontaneous precipitation of crystalline protein appeared first after 24 hours and amounted to 0.6 Gm per cent of plasma. The electrophoretic pattern of this plasma showed 75.6 per cent of the total area in the gamma globulin peak. This is reminiscent of the case of Holmberg<sup>5</sup> in which precipitation could be prevented by anaerobic collection. The stimulus to precipitation seemed to be the loss of CO<sub>2</sub> on exposure to air. Olhagen did not find anaerobic collection a factor in his Case II and does not suggest a cause for the protein precipitation. Olhagen's Case III (probably "a myeloma diagnosis in spite of the negative marrow and x-ray findings") was complicated by renal insufficiency and high blood nonprotein nitrogen. There was no hyperproteinemia (total protein 7.4 Gm per cent). The spontaneously crystallizing protein amounted to 0.6 Gm per cent of plasma; it precipitated immediately on cooling, and redissolved promptly on warming to 37° C. A urinary protein was electrophoretically identical with the cryoglobulin of the plasma.

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Wertheimer and Stern<sup>7</sup> were the first to suggest a classification of "cold fraction" plasma proteins. They made a systematic search for such proteins following their discovery of a "cold fraction" in the plasmas of dogs infected with kala-azar. "Cold fractions" were classified by them as Grades 1, 2, 3, 4, and 5. Grade 1 was defined as including those plasmas showing "turbidity marked but no precipitate" after standing twenty-four hours in the refrigerator at 7 to 11° C, Grades 2 and 3 were intermediate and Grade 4 included plasmas showing a "marked precipitate easily estimable by quantitative methods." Grade 5 was reserved for reversible cold fractions of a nonprotein nature, usually cholesterol, of which these authors found a considerable number. Investigators are warned against mistaking Grade 5 cold fractions for cold-sensitive proteins. Five cases of kala-azar in human beings were investigated, two of which showed Grade 4 cold fractions while the other three showed Grade 3 precipitations. This indicates that large quantities of cold sensitive plasma proteins may be a common occurrence in this disease to be found for the searching. Eight cases of endocarditis lenta all showed cold fractions of Grades 1 to 3, with one showing Grade 4. Grades 1 to 3 were found in four cases of nephrosis. In the twenty-seven cases studied, Grade 5 precipitates (nonprotein reversible cold fractions) were found in six.

A more extensive and more systematic search for cold-sensitive plasma proteins was reported by Lerner, Barnum, and Watson<sup>8</sup> (1947). These authors examined the serums from one hundred twenty-one patients with a variety of pathologic conditions and from forty controls. Their work is the basis for a very practical classification of cryoglobulins according to the time of appearance on standing at 5° C and according to the amount of precipitate formed. Grade 1 includes plasmas containing a trace to 6 mg per cent of cryoglobulin appearing between twenty-four hours and one week, Grade 2 includes plasmas containing 6 to 25 mg per cent appearing in twenty-four hours, Grade 3 includes plasmas containing more than 25 mg per cent of cryoglobulin appearing immediately on cooling. In one hundred twenty-one cases examined they found twelve cases of Grade 2 and eighteen cases of Grade 1 cryoglobulinemia. None were found among the forty controls. No case of Grade 3 cryoglobulinemia was found because of the systematic search, the one case listed was the first seen by these authors and was apparently the stimulus for the later work. The behavior of lipid precipitates was found to be "entirely different from the cryoglobulins." It should be emphasized that the Grade 3 cryoglobulinemia of these authors, the grade including the heaviest precipitation, corresponds to Grade 4 of Wertheimer and Stern<sup>7</sup>. In our search of the literature, we have found, including our own, fifteen cases of cryoglobulinemia that would be classified as Grade 3 according to the classification of Lerner, Barnum, and Watson.<sup>8</sup>

#### CASE REPORT

In the present paper we are reporting some properties of the plasma and of a cryoglobulin separated from the plasma of a person (Patient O. O.) suffering from multiple myeloma. The patient, a white woman, 55 years of age, entered Colorado General Hospital



Fig 1—X ray of left shoulder showing extensive destruction of head of left humerus and osteolytic process in the left scapula and clavicle.

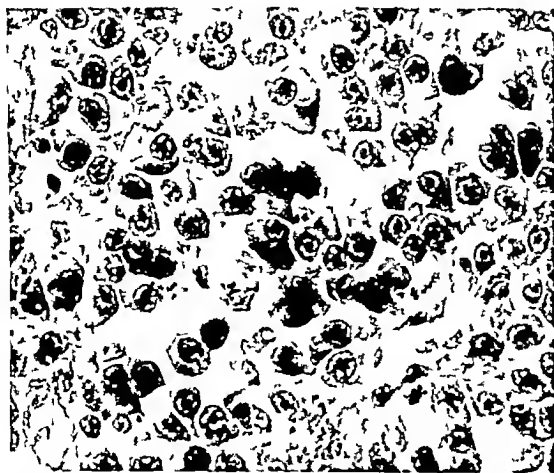


Fig 2—Plasma cell myeloma of left humerus. Note prominent nuclei often eccentric, sometimes double with heavy coarse margined chromatin and large conspicuous nucleoli. Hematoxylin and eosin stain. ( $\times 600$ )

on Aug 7, 1945 For the past seven years she had suffered from severe sensitivity to cold Death occurred the following September 9 Autopsy revealed plasma cell myeloma involving the bone marrow, chiefly of the left humerus, scapula, and clavicle The bone of the head and neck of the humerus was extensively destroyed by massive proliferation of cells with the morphology of plasma cells, chiefly in the plasmoblast stage (Figs 1 and 2)\*

#### RESULTS

*Properties of the Plasma*—When the blood of Patient O O was first drawn it was found to solidify quickly in the needle and syringe even though large amounts of anticoagulant were used However, when the syringe and needle were warmed before use and the sample of drawn blood was kept warm, it remained liquid indefinitely with no more than the usual additions of anti-coagulant When heparinized blood was kept at 37 to 40° C throughout the process of centrifugation it separated into three layers an upper layer of apparently normal plasma, a middle, clear, almost water-white layer, and a lower layer of cells On cooling, the upper layer remained liquid The middle layer, however, congealed to a pearly white translucent solid In a sample of 75 ml of blood, the volumes of the three layers were upper, 30 ml, and middle, 28 ml, and lower, 17 milliliters Thus the middle layer of cold sensitive material accounted for 37 per cent of the total volume of the blood When the middle layer solidified on cooling the top layer could be poured off and, with distilled water, could be washed off completely from the surface of the solid middle portion On warming, the middle layer again liquefied and could be pipetted from the surface of the cells This separation was accomplished on five different samples drawn on five different days Following a whole blood transfusion the separation into three layers never again occurred though the whole blood or plasma solidified on cooling as before After dilution with distilled water the cryoglobulin of these latter samples separated on cooling first as a thin gel which then quickly broke up as a flocculent precipitate and could be purified by resolution in warm water from which it again precipitated on cooling Solidification on cooling to about 32° C and liquefaction on rewarming to 38° C was repeated on the same sample of whole blood fifteen times without any apparent changes in its properties

Fractional plasma protein analyses were carried out by means of a micro-modification<sup>10</sup> of the method of Howe These and other analytic data are collected in Table II The urine was free from protein at all times

In dilute solution the cryoglobulin was precipitated solely in the globulin fraction This was not true in high concentrations and it is probable that the high 'fibrinogen' fraction on Aug 28, 1945, contained a large amount of cryoglobulin

The viscosity of the plasma was measured at different temperatures with the Ostwald viscosimeter Exactly 3 ml of the plasma were used in the instrument which was then placed in a water bath at 45° C and allowed to stand for thirty minutes to come to thermal equilibrium After several determinations of viscosity were made at this temperature, the bath was cooled quickly with crushed ice and a new temperature level established By repeating this procedure, the data for viscosity changes with temperature were obtained

\*The pathologic aspects of the case have been reported\*

In Fig. 3 the curve for the plasma of Patient O O is compared with the average curve for three normal plasmas measured in this laboratory and the average curve for three normal plasmas and the curve for one hyperproteinemic plasma (9.2 Gm per cent) reported by Jochims.<sup>11</sup> The viscosity of the plasma of Patient O O is more than five times that of the average normal plasma at 38° C and rapidly increases to infinity at 32° C. If the viscosity of normal

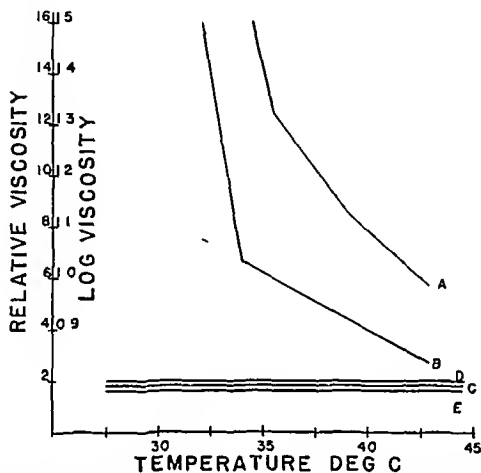


Fig. 3—The changes of viscosity with temperature of the plasma of Patient O O. A case of hyperproteinemia reported by Jochims. D the average of three normals reported by Jochims (C) and F the average of three normals examined in this laboratory. Curve B is the log of the viscosity of the plasma of Patient O O showing a break at about 34° C.

plasma is plotted against the logarithm of the temperature, a continuous straight line is obtained. When the viscosity of the plasma of Patient O O is so plotted, two straight lines are obtained. The position of the break in the semilogarithmic curve suggests that the change in state of the cryoprotein on cooling begins when the temperature reaches 34° C.

**Chemistry of the Cryoglobulin**—A large sample of whole plasma was diluted and cooled in the icebox for several hours. It was then centrifuged with the centrifuge tubes pre-cooled in ice. The cryoglobulin separated in this way was quickly and completely dissolved when placed in warm (40° C) distilled water. The solution was again cooled in the icebox to reprecipitate the cryoglobulin. In this way the sample was purified by ten reprecipitations from distilled water. A small sample of the final product was dried to a pure white powder which gave strong biuret, xanthoproteic, Millon, and Acree-Rosenheim tests and a negative Molsch test. The bulk of the product was redissolved in warm distilled water and mixed with an equal volume of

95 per cent ethyl alcohol and placed in the icebox. Amorphous material began to precipitate at once and after three days needlelike crystals appeared (Fig 4). These were at first presumed to be crystals of the cryoglobulin because of the similar crystals found by Wintrobe and Buell<sup>1</sup> and the protein crystals reported in the case of myeloma of von Bonsdorff, Groth, and Packalen.<sup>12</sup> However, the crystals proved to be not protein but cholesterol ester (method of Schoenheimer and Sperry<sup>13</sup>) with no free cholesterol and no



Fig 4—Crystals of cholesterol ester separated from purified protein after standing in the cold room three days in 50 per cent ethyl alcohol ( $\times 60$ )

nitrogen or phosphorus present. The crystals must represent a "lightly bound" lipid fraction in the protein molecule because although they were not removed by ten resolutions in and precipitations from distilled water, they were removed by treatment in the cold for three days with 50 per cent ethyl alcohol. The quantity of crystals was approximately 2 per cent of the cryoglobulin.

The remaining amorphous protein was dried and subjected to repeated extraction with a 1:2 mixture of ethyl alcohol and ethyl ether. This treatment yielded an additional 0.17 per cent of material which proved to be 20 per cent cholesterol ester and 70 per cent fatty acid. Again no free cholesterol was present and only a trace of lipid phosphorus.

Four samples of the extracted and dried protein were subjected to micro-Kjeldahl analysis<sup>10</sup> and were found to contain 15.2, 15.2, 15.2, and 14.9 per cent nitrogen respectively, with an average of 15.13 per cent. Phosphorus and purines were not present in detectable quantities.

Another sample was assayed for amino acids by microbiologic methods essentially those of Stokes, Gunness, Dreyer, and Caswell<sup>14</sup> and Stokes and Gunness.<sup>15</sup> Nine amino acids were determined with *Streptococcus faecalis* (American Type Culture Collection No. 9790) and the tenth, phenylalanine with *Lactobacillus delbrueckii* LD 5 (American Type Culture Collection No. 9595).

In Table I these assays are compared with assays, taken from the literature of some normal plasma protein fractions and with Bence Jones protein. The cryoglobulin shows an amino acid pattern suggesting relationship to the beta or gamma globulins.

TABLE I. AMINO ACID ANALYSES OF CRYOGLOBULIN COMPARED WITH ANALYSES OF NORMAL PLASMA PROTEINS AND BENCE JONES PROTEIN

AMINO ACID	CRYO GLOBULIN (GM %)	BENCE JONES PROTEIN (GM %)	ALBUMIN   (GM %)	β-GLOBULIN   (GM %)	γ GLOBULIN   (GM %)	FIBRINO GEN (GM %)
Histidine	1.96	1.1 1.4	3.5	2.50	2.50	2.8
Arginine	5.56	5.2 5.0†	6.15	5.64	4.80	7.9
Lysine	7.28	7.0 6.5†	5.8		6.7	
Tryptophane	1.59	2.5 2.5†	0.19	2.06	2.88	3.22
Valine	9.60	5.6‡				
Leucine	8.70	6.5‡ 10.6§	11.9	8.9	9.32	7.1
Isoleucine	2.13					
Phenylalanine	3.99	4.9 1.5§				
Methionine	.90		1.28	1.54	1.06	2.52
Threonine	5.88		5.03	7.26	8.36	6.6

First case of Calvery and Freyberg

†Second case of Calvery and Freyberg \*

‡Hopkins and Savory (average of two cases)

§Abderhalden and Rostok

||Brand, Hassell and Said 19

All of the analyses the physical properties and the behavior toward Na<sub>2</sub>SO<sub>4</sub> fractionation of this cryoglobulin are compatible with its classification as a pseudoglobulin. It differed from other cryoproteins that have been described in its higher concentration in the plasma and the higher temperature at which it precipitated on cooling. After removal of the cryoglobulin the remaining plasma appeared to be qualitatively normal though the albumin concentration was low and the globulin and fibrinogen were high (Table II).

TABLE II. PLASMA PROTEIN AND OTHER LABORATORY FINDINGS ON CRYOGLOBULIN CONTAINING PLASMA OF PATIENT O O

PLASMA	DATE				
	8/11/45		8/24/45		8/-8/45
	WHOLE PLASMA	WHOLE PLASMA	UPPER LAYER	MIDDLE LAYER	WHOLE PLASMA†
Total protein (Gm %)	7.2	10.0	7.1	13.1	18.1
Albumin (Gm %)	2.2	1.8	2.1		2.1
Globulin (Gm %)	5.0	6.2	4.5		9.8
Fibrinogen (Gm %)		1.7	0.5		0.2
N P N (Mg %)	27	29	37		27
Prothrombin (% normal)	44				
Total cholesterol (Gm %)	146				122
Serum Ca (Gm %)	10.4				10.4
Serum P (Gm %)	4.2				7.1
Alk. phosphatase (Bodansky units)	2.4				2.4
Coll. agglutinins	Neg				
WHOLE BLOOD	DATE				
	8/11	8/-0			8/-8
Hemoglobin (Gm %)		5.5			4
Erythrocytes	3,500,000	1,950,000			
Leucocytes	0.700	7.200			
Segmented neutrophils	.48	.38			
Lymphocytes (%)	.50	.61			

Globulin plus fibrinogen.

†A whole blood transfusion was given on 8/6

# IMMUNOCHEMICAL ESTIMATION OF THE RATE OF DISAPPEARANCE OF TRANSFUSED GAMMA GLOBULIN FROM THE BLOOD IN TWO CASES OF HYPOPROTEINEMIA

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SEVERAL cases have been reported in which an unusually low serum gamma globulin was observed electrophoretically<sup>1-5</sup>. In two of these, reported in detail elsewhere,<sup>3, 4</sup> an opportunity was presented to follow by immunochemical methods the disappearance of gamma globulin from the blood after a transfusion of plasma. The quantitative precipitin method has been used by several investigators for the estimation of various serum proteins<sup>6, 7</sup> and more recently a micromodification<sup>8</sup> has been applied to the determination of the gamma globulin and crystallizable albumin in human cerebrospinal fluid<sup>10</sup>. The latter technique was applied in this study and indicated a "half-life" of 3 to 4 days for gamma globulin.

In one of the patients, the ability to form antibodies was tested by subcutaneous injection of blood group A substance<sup>11, 12</sup> and of a mixture of six type-specific pneumococcal polysaccharides<sup>13, 14, 15</sup>. The degree of utilization of dietary amino acids for tissue synthesis was evaluated in the second patient by determining the rate of excretion of N<sup>15</sup> in the urine after ingestion of a known amount of isotopic glycine.<sup>16</sup>

## EXPERIMENTAL

One patient, E. B., was a 31 year old woman. Her case history has been presented by Homburger and Petermann<sup>3</sup> in connection with a detailed description of a new syndrome, familial idiopathic hypoproteinemia. Despite a constant high protein diet, the gamma globulin level remained low. No evidence of inefficient utilization of dietary protein could be found. Proteinuria was absent. The rate of synthesis of serum protein was found to be normal.<sup>3</sup> The general clinical picture was negative.

A small blood sample (E. B.) was taken before the intravenous injection of 975 ml of normal donor plasma. Blood samples were then removed two hours later and after one, three, five, and nine days.

Determination of the gamma globulin in the transfused plasma and in the serum samples was carried out as previously described<sup>10</sup> except for the addition of appropriate dilutions of the serum samples to 10 ml portions of calibrated rabbit antiserum to purified and electrophoretically homogeneous human gamma globulin (gamma globulin of Deitch and co-workers<sup>17</sup>) and measuring the quantities of specifically precipitable nitrogen. The equivalent amounts of gamma globulin are found by interpolation on the calibration curve. Dilutions of the serum samples of 1:50 to 1:200 were required to ensure that all analyses were carried out in the region of antibody excess (as is necessary for the immunochemical method to be valid). The results are listed in Table I.

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TABLE I. DISAPPEARANCE OF GAMMA GLOBULIN ON INJECTION OF NORMAL PLASMA

BLOOD SAMPLE	NUMBER OF DAYS AFTER TRANSFUSION	GAMMA GLOBULIN PER ML. (MG)	TOTAL PROTEIN PER 100 ML. (GM)
<i>Latent E B</i>			
Donor plasma		12.4	6.15
E B		3.4	5.31
E B <sub>1</sub>	(2 hr)	6.6	5.49
E B <sub>2</sub>	1	6.1	6.01
E B <sub>3</sub>	3	5.2	5.83
E B <sub>4</sub>	5	4.5	5.27
F B <sub>5</sub>	9	3.1	5.13
<i>Patient B V</i>			
Donor plasma		11.5	5.84
B V		3.1	3.58
B V <sub>1</sub>	(2 hr)	5.6	4.36
B V <sub>2</sub>	1	4.6	3.90
B V <sub>3</sub>	3	4.1	3.81
B V <sub>4</sub>	5	3.4	
B V <sub>5</sub>	9	3.6	3.56
B V	14	3.0	3.28

Subscripts x Preinjection level 1 Level in blood sample taken two hours after injection of plasma.

To determine the antibody response on injection of hog blood group A substance, the patient (F B) received subcutaneously on two occasions two days apart a total of 2 mg of a purified hog stomach blood group A preparation (preparation 2A<sup>11</sup>). Examination of a blood sample obtained ten days later revealed no significant change in antibody level.

The patient also received an 0.8 ml injection of a mixture of pneumococcal I, II, III, V, VII, VIII type specific polysaccharides which also contained some group specific C<sup>12</sup> substance.\* Analyses were performed<sup>14</sup> on bleedings taken just before and three weeks after the immunization. The results are listed in Table II.

TABLE II. ANTIBODY LEVEL IN E B AFTER INTRACUTANEOUS INJECTION OF SPECIFIC POLYSACCHARIDES OF TYPES I, II, III, V, VII, AND VIII

	ANTIBODY N PER 4 ML. SERUM						
	ANTIBODIES TO						
	C	I	II	III	V	VII	VIII
Before	0.003 µg	0 µg	0 µg	0 µg	0 µg	0 µg	0 µg
After immunization	0.003 µg	0.001 µg	0.005 µg	0 µg	0 µg	0.018 µg	0.004 µg

The second patient B V, a 33 year old female mechanic, showed a generalized nonspecific lymph node enlargement in 1937 which was radiosensitive and disappeared on treatment in August, 1938. The albuminuria noticed in 1938 did not reappear. With the exception of edema of the lower extremities the clinical picture was negative. An intake of 90 Gm of protein per day was required to maintain nitrogen balance. For fifteen days before and for the duration of the experiment (Jan 9 to 23, 1948) the daily protein intake was about 1.0 Gm per day. A detailed case history will be given elsewhere.<sup>4</sup> The patient died on March 13, 1948, from a brain hemorrhage, and a giant follicular lymphoma was found on autopsy.

One half liter of normal plasma was injected intravenously after a blood specimen (B V) had been taken. Additional blood specimens were removed two hours, one, three, five, nine and fourteen days later for total protein and gamma globulin analyses. As in the case of E B, serum samples prepared from the bleedings were diluted appropriately. The gamma globulin determinations are listed in Table I. Duplicate analyses were performed throughout.

Manufactured by E. R. Squibb and Co. New Brunswick, N. J.

\*The vaccine was obtained through the kindness of Professor Michael Heidelberger.

†By Mrs. Marie DiLapi in Dr. Heidelberger's laboratory.

To obtain information concerning the efficiency of utilization of dietary glycine,<sup>16</sup> B V received orally 540 mg of glycine<sup>18</sup>\* containing 32 per cent excess N<sup>15</sup>. Total daily urine collections were made for three days. Isotope determinations† indicated that 42, 52, and 60 per cent of the dietary isotope had been eliminated twenty four, forty eight, and seventy two hours after ingestion.

### RESULTS

From Table I it may be seen that injection of the plasma caused almost a doubling of the gamma globulin concentration in the blood serum of both subjects, although there was a very much smaller increase in total serum protein. In both instances, it required about nine days for the gamma globulin to return to the preinjection level, and about three to four days for the initial increase in gamma globulin to be reduced by one-half, indicating a "half life" for the injected gamma globulin of about 3 to 4 days.

As for the response of E B to injection of pneumococcal specific polysaccharides, the data in Table II reveal no measurable antibody to types III and V and extremely little to type I. The greatest response was to type VII. The values for types II and VIII are probably significant.

### DISCUSSION

Although many electrophoretic studies on plasma proteins have been carried out, only very few instances of low gamma globulin have been recorded. Perhaps the first record is that by Forbes in 1944<sup>1</sup> of a hypoproteinemic woman. The gamma globulin studied electrophoretically was unaffected by ingestion or intravenous injection of Anugen. Following a plasma transfusion, this patient eliminated most of the plasma protein she had received intravenously in eight days. Kiebs<sup>2</sup> described a 15-year-old girl suffering from malnutrition who showed a general hypoproteinemia with an electrophoretically determined gamma globulin level of 0.15 Gm per 100 ml plasma. This returned to about normal (0.65) on dietotherapy. Two other cases of hypogammaglobulinemia, in children, have been cited by Stern.<sup>3</sup>

In the present study, an attempt has been made to consider the possible metabolic basis for the low gamma globulin levels. It has been seen that a decreased gamma globulin due to low protein intake may be restored to normal when the proper protein intake is maintained.<sup>2</sup> This explanation obviously does not apply to the two patients studied, for they were kept on protein intake levels far in excess of that required for nitrogen balance without affecting their serum gamma globulin.

The present study showed that on transfusion of an amount of plasma sufficient to double the circulating serum gamma globulin, this component returned to its original level at a rate indicating a "half-life" of 3 to 4 days. Unfortunately, the procedure does not lend itself to a comparison in normal subjects since it would not be possible to raise the gamma globulin level significantly by transfusing normal plasma into a normal subject. It therefore becomes necessary to compare the "half-life" of gamma globulin

\*Obtained through the kindness of Dr. David Rittenberg.

†Carried out by Mr. I. Sucher in Dr. Rittenberg's laboratory.

found by this method with that found by other methods. A "half life" of about two weeks has been found for human serum globulin by isotope studies.<sup>13</sup> <sup>1</sup> footnote, p. 317

The specificity of the immunochemical method, as applied to the estimation of gamma globulin has been considered previously.<sup>10</sup> Deutseh and co-workers<sup>10</sup> have found extensive cross reactions with human gamma<sub>1</sub> and gamma<sub>2</sub> globulin. Very recently it has been reported<sup>20</sup> that several preparations of human gamma<sub>2</sub> globulins react alike with rabbit anti gamma<sub>2</sub> serum. Since immunochemical determinations of serum gamma globulin were consistently higher than the electrophoretic values Jager, Smith, Nickerson and Brown<sup>20</sup> inferred that other serum proteins (probably globulins) cross reacted with the antiserum. The individual values obtained in this investigation therefore probably represent all constituents reactive with antibody to gamma<sub>2</sub> globulin. There is however general agreement between the values reported here and those for gamma globulin obtained on the same serum samples (from E. B.) by electrophoresis,<sup>3</sup> from which the same "half life" can be calculated.

To determine antibody production one of the patients (E. B.) received injections of the blood group substance<sup>11, 12</sup> and several type specific pneumococcal polysaccharides.<sup>13, 14, 15</sup> There was no response to the A substance and very little or no anti SI, SIII and SV formed. The patient did however form antibodies against types II, VIII and VII although the antibody levels reached were not striking. These data while below average do not differ sufficiently to be abnormally low. It might be of interest in this connection to compare these findings with the report<sup>1</sup> that rabbits showed a lower capacity to produce agglutinins against *Escherichia typhosa* when made hypoproteinemic by low protein diets or plasmapheresis.

When the high protein diet of the second patient (B. V.) is taken into account, the rate of excretion of isotope in the urine after ingestion of a single small dose of isotopic glycine<sup>16</sup> suggests a rather normal utilization for protein synthesis.

#### SUMMARY

1. The rate of elimination of transfused gamma globulin from the blood stream of two patients with idiopathic hypoproteinemia was followed immunochemically by the quantitative precipitation method.

2. The "half life" of transfused gamma globulin in these patients was found to be three to four days.

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## SENSITIZATIONS TO THE FACTOR Rh IN NEGROES

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THE literature on Rh sensitizations in Negroid races is exceedingly sparse, and with the exception of one report by Wiener and Wexler<sup>1</sup> no systematic study of this problem has come to our attention. In that report, three cases of erythroblastosis fetalis in Negroes are described, none due to Rh and two due to apparent sensitizations against the factors A or B. The inference is made that in this group of people there might be a better placental barrier or there may exist a lower sensitivity to the antigen Rh. In a subsequent personal communication to one of us (R. K. W.) it was pointed out that the intentions of that paper were actually to report three unusual cases and to arouse interest in the subject matter and not to make any generalized statements concerning Rh sensitizations in Negroes.

It is the purpose of this paper to describe our experience with isoimmunization in Negroes taken at random from the obstetric clinic of the Medical College of Virginia.

### MATERIALS AND METHODS

During the past two years 3115 consecutive obstetric clinic patients were examined for blood groups Rh factor and presence of isoantibodies. Those found to be negative with anti D (Rh<sub>0</sub>) serum were checked at approximately monthly intervals for antibodies. Whenever possible, specimens of cord blood were obtained at the time of delivery. Presence of antibodies was determined simultaneously by the saline agglutination, 20 per cent bovine albumin, and antiglobulin<sup>2</sup> techniques. The cord specimens were grouped and typed, the cells studied with the antiglobulin technique for antibody coating and the respective sera searched for antibodies with the methods mentioned. The children born of immunized Rh negative mothers were checked with the routine hematologic methods as well as the antiglobulin technique.

### RESULTS

As shown in Table I over 86 per cent of patients were Negroes. The incidence of d (Rh<sub>0</sub> negative) in Negroes and Caucasians was 73 and 17.4 per cent respectively. Among the 197 Rh negative Negroes, eight were found to be sensitized to Rh and among the seventy three Caucasians three were shown to have Rh antibodies. If one expresses these figures in per cent of d (Rh negative) (see Table II) one finds that 4.06 per cent of the Negroes and 4.1 per cent of the Caucasians were sensitized. However in relating the number of sensitized patients to the total number in each racial group it may be

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TABLE I

	NEGROES	PER CENT	CAUCASIANS	PER CENT
D (Rh <sub>0</sub> pos)	2499	92.7	346	82.6
d (Rh <sub>0</sub> neg)	197	7.3	73	17.4
	2696		419	

shown that 0.3 per cent of the Negroes and 0.7 per cent of the Caucasians had developed Rh antibodies. The ratio of Negro to white Rh negatives in this series is 1.24, and the corresponding ratio of sensitized patients, 1.27.

TABLE II

d (Rh <sub>0</sub> NEGATIVE)	NEGROES	CAUCASIANS
Total number of Rh negative patients	197	73
Patients sensitized	8	3
Per cent sensitized	4.06	4.1
Per cent sensitized of entire racial group	0.3	0.7

As shown in Table III, all of the Negro patients were multiparas. One subject (Patient 2) had a definite history and another (Patient 4) a questionable history of erythroblastosis in a previous pregnancy. In four of the eight cases a history of transfusion was elicited. Only Patient 6 received transfusions preceding the first pregnancy. All but one (Patient 8) showed a consistent absence of Rh agglutinins. In five patients the outcome of the pregnancy is known, and at the time of birth all were described as clinically

TABLE

PATIENT	RACE	PARITY		PREVIOUS INSTANCE OF ERYTHROBLASTOSIS FETALIS	TRANSFUSION HISTORY	MATERNAL RED CELLS		ANTI Rh			TITERS	CLINICAL APPEARANCE AT BIRTH
		PARA	GRAVIDA			GROUP	Rh TYPE	SERUM LINE	ALBUMIN	ANTI GLOBULIN		
1	Negro	II	III	—	1	O	rh''(dce)	0	64	128	3rd	Normal
2	Negro	XII	XIV	11th, 12th, and 13th children	—	O	rh(dce)	0	8	128	3rd	Normal
3	Negro	I	III	—	—	O	rh(dce)	0	16	32	3rd	Normal
4	Negro	III	IV	3rd child jaundiced	—	O	rh(dce)	0	16	16	3rd	Normal
5	Negro	III	VI	—	4	O	rh(dce)	0	4	4	3rd	Normal
6	Negro	VIII	VIII	—	6*	B	rh(dce)	0	16	16	3rd	
7	Negro	I	III	—	1	O	rh(dce)	0	16	16	3rd	
8	Negro	I	II	—	—	B	rh(dce)	4	16	16	3rd	Mild jaundice
9	Caucasian	V	VIII	—	—	O	rh(dce)	Trace	8	8	3rd	Normal
10	Caucasian	II	III	2nd child	—	A	rh(dce)	0	8	32	3rd	
11	Caucasian	III	IV	2nd and 3rd children	—	O	rh(dce)	0	32	32	1st	

\*Previous to first pregnancy.

†These cells were saturated with incomplete maternal antibody so that they failed to be agglutinated in vitro by anti-Rho agglutinating sera.

normal. There was not a single instance of incompatibility of the major blood groups. However, on laboratory examination the four cord and/or peripheral blood specimens belonging to Rh positive children showed antibody coating to a varying extent (antiglobulin technique).

## CASE REPORTS

CASE 1—The clinical appearance of this infant at birth was that of an entirely normal full term baby. Its red blood cells however were 'blocked' so that they gave the reaction of Rh<sub>+</sub> negative cells with agglutinating sera. The antiglobulin reagent gave a 4+ reaction on cord and peripheral cells and incomplete antibodies could be demonstrated in the cord serum. Hemoglobin and red blood count shortly after birth were 13.6 grams and 4.3 million respectively. This child was discharged on the third postnatal day because of 'apparent' lack of involvement. The subsequent course is summarized in Table IV.

As can be seen from Table IV this infant's hemoglobin dropped markedly after its discharge and the infant was brought back to the hospital only because the mother had been warned of the possible complications. There was no evidence of jaundice at any time. Despite four transfusions, the hemoglobin value persistently dropped as long as antibodies could be demonstrated to be attached to the red cells (i.e. approximately seven and one half weeks postpartum).

CASE 2—This infant was apparently normal at the time of delivery but on the eighth day was noticed to be jaundiced, pale and in acute respiratory distress. On admission hemoglobin was 2 grams and red blood count 0.10 000 per cubic millimeter. Erythrocytes gave a 4+ antiglobulin reaction but were not completely 'blocked'. Incomplete anti-Rh antibodies could be demonstrated in the serum. The subsequent course is summarized in Table V.

## III

BABY'S BLOOD		CORD BLOOD		PERIPHERAL BLOOD			COURSE
GROUP	Rh TYPE	ANTI GLOBULIN TEST ON CELLS	ANTI BODIES IN SERUM	ANTI GLOBULIN	HGB	RBC	
O	blocked <sup>†</sup>	4+	Present	4+	13.6	4.3	Transfused 4 times, now living and well
O	Rh (Dce)			4+	2.0 (8th day)	0.6	Transfused 4 times now living and well
O	blocked <sup>†</sup>	3+	Present	3+	12.0	3.8	Replacement transfusion, now living and well
O	rh (dCe)			Neg	12.6		Living and well
O	Rh <sub>+</sub> (Dce)	1+	None	1+	15.0		50 days post partum Hgb 10 Gm, antiglobulin negative now living and well
		Undelivered					Living and well
O	Rh <sub>+</sub> (Dce)	Outcome unknown	None	1+	Normal limits		Transfused once now living and well
AB	Rh (Dce)	3+	None	→	17.0		14 days post partum Hgb 9.8 h BC 31 antiglobulin 1+ transfused once, now living and well
		Undelivered					

TABLE IV CASE 1

POST NATAL DAY	ANTI GLOBULIN TEST	HGB (GM)	RBC (MIL LIONS)	WBC	NORMO BLAST	RETICU LOCYTES	COMMENTS
1	4+	13.6	4.3	21,600	9%	8.6%	
2	4+	13.6					
3	4+	13.7					
16	4+	8.8	2.8				Discharged
17	4+	10.7					Readmitted, 85 c.c.
20	4+	10.2					transfusion
21	4+	12.0					85 c.c. transfusion
22	4+	14.8					85 c.c. transfusion
23	4+	14.0					
24	4+	13.6					
25	4+	13.6					
27	4+	17.0	5.4	5,300	0		
51	1+	9.9					
71	Neg	11.0					

As in the first infant, the intensity of the antibody coating of the red cells seemed to parallel the clinical course. It is of interest that the mother of this child lost her four previous children from erythroblastosis. Her ninth and tenth children were severely jaundiced and lived for six and twelve days respectively. Her eleventh and twelfth pregnancies terminated in nine months with stillbirths.

TABLE V CASE 2

POSTNATAL DAY	ANTIGLOBU LIN TEST	HGB	RBC	COMMENTS
8	4+	2.0	610,000	150 c.c. transfusion (whole blood)
9	1+	2.0		35 c.c. washed cells
10	1+	14.0		
11				70 c.c. washed cells
16	3+	18.8		Patient dehydrated
19				120 c.c. whole blood
21	Neg			
29	Neg			Peripheral blood appeared entirely Rh negative
31	1+	13.5		Patient clinically well
38	Neg	13.0		Discharged, living and well since

CASE 3—This infant also was described as clinically normal at birth. Its hemoglobin was 120 grams, with a red blood count of 3.8 million per cubic millimeter. Antiglobulin reaction was 4+. Red cells from the cord as well as from the baby's peripheral circulation were completely "blocked" and the cord serum contained incomplete antibodies. Because of our experience with previous similar cases, a replacement transfusion of 500 c.c. was given on the first postnatal day. Antiglobulin reaction after the transfusion was 1+. On the third day hemoglobin had risen to 16.4 grams with a weakly positive antiglobulin test. Thirty days after the replacement transfusion the baby was discharged from the hospital with a hemoglobin of 11.2 grams, red blood count of 4.0 million per cubic millimeter, and a negative antiglobulin reaction. The baby has remained well since.

CASE 4—This child was clinically Rh<sub>0</sub> negative (dCe, rh') and consequently not affected by the maternal antibodies which were anti D (Rh<sub>0</sub>) in specificity. Antiglobulin reaction on cord cells was negative and the infant remained well despite a somewhat low hemoglobin (12.6 grams) at birth. It is of interest that on a six week post partum sample of the mother's blood, anti rh' (anti C) agglutinins could be demonstrated.



CASE 5—This baby was born of a mother possessing a very low incomplete Rh antibody titer. At the time of birth the baby appeared normal and has remained so. Its hemoglobin was 15.6 grams and the antiglobulin reaction on cord cells was only 1+. There were no free antibodies demonstrable in the cord serum. On the fiftieth post partum day the hemoglobin had dropped to 10.0 grams and no antibodies could be detected on the surface of the red cells.

CASE 6—As yet undelivered.

CASE 7—This was a normal full term child which has remained well according to the mother. This child was not available to us for testing.

CASE 8—The outcome of this case is unknown to us but because of low parity and the low titer of incomplete antibodies in the third trimester, experience indicates that this child probably was not affected.

CASES 9, 10, and 11—These are the cases observed in Caucasians and are listed only because they occurred within our present series.

### DISCUSSION

Because of our findings that Negroes can be apparently immunized against Rh with the same frequency as Caucasians we proceeded to check our hospital statistics for clinical erythroblastosis in the two races over a period of eight years. These findings are summarized in Table VI.

TABLE VI

YEAR	NUMBER OF HOSPITAL CASES OF ERYTHROBLASTOSIS FETALIS		TOTAL HOSPITAL BIRTHS	
	CAUCASIANS	NEGROES	CAUCASIANS	NEGROES
1941	0	0	697	639
1942	0	0	828	823
1943	0	0	980	850
1944	5	0	1,064	825
1945	5	1	1,267	952
1946	2	0	1,490	1,222
1947	7	1	1,649	1,522
1948	10	5	1,460	1,870
Total	29	7	9,500	8,931

From Table VI it can be seen that over the eight year period erythroblastosis was diagnosed in Caucasians at a rate of 1 out of approximately 300 births. For the Negroes this figure, however, was only 1 in 1,300 cases. The ratio of frequency between the two groups is 1:4. It can also be seen that as the years passed the frequency of the diagnosis rose with increasing knowledge of the Rh factor. The majority of cases have been diagnosed during the past two years over which period our study has extended.

The incidence of erythroblastosis reported by various authors ranges from 1/150 to 1/568 (see Table VII) with an average incidence of about 1/300+. However it is to be noted that in most statistics cited the Negro cases have been included in the total group.

TABLE VII INCIDENCE OF ERYTHROBLASTOSIS

AUTHORS	YEAR	INCIDENCE
Diamonds <sup>3</sup>	1945	1/150
Ionder Ponder and Levine <sup>4</sup>	1948	1/169
Schwartz and Levine <sup>5</sup>	1943	1/200
Lotter <sup>6</sup>	1947	1/252
Javert	1942	1/438
Wolfe <sup>8</sup>	1940	1/568

In order to ascertain whether the difficulty in finding cases of erythroblastosis in Negroes was shared by other institutions, a questionnaire\* was sent to seven departments of pediatrics in regions with large Negro populations. Four of these institutions reported cases and gave the frequency of Negro births. These are summarized in Table VIII.

TABLE VIII

	CASES OF ERYTHROBLASTOSIS FETALIS, HYDROPS, OR ICTERUS GRAVIS IN NEGROES	DUE TO Rh?	NUMBER OF NEGRO BIRTHS
1 Duval County Hospital, Jacksonville, Fla (H A Carithers)	None	—	488 per yr
2 Tulane University, New Orleans, La (R V Platou)	5	Yes, 2 due to AB incompatibility	6,000 per yr
3 Duke University, Durham, N C (G Taylor)	1	Yes	535 per yr
4 Louisiana State University, New Orleans, La (M E Wegman)	3	Yes, also Hr	6,500 per 2 yr

\*Through courtesy of Dr Lee E Sutton, Jr Professor of Pediatrics Medical College of Virginia

In this survey seven cases are reported out of 13,523 births if one takes into account only those hospitals which stated their incidence of Negro births and only those cases which were proved to be due to Rh incompatibility. This frequency of about 1 in 1,900 births approximates the incidence in our institution (1/1,300).

It remains to be explained why only one case of erythroblastosis is diagnosed in 1,300 births if the rate of immunization to the factor Rh among Negroes is 1 in approximately 330 (0.3 per cent).

As stated by Levine,<sup>9 10 11</sup> the true incidence of erythroblastosis fetalis in any given population can be expected to be proportional to the frequency of negative reactions with anti-D (Rh<sub>0</sub>) serum. That this apparently holds for immunizations against the Rh factor can be seen from our figures (see Tables I and II). The reasons for the failure to find the expected number of cases of erythroblastosis in Negro infants are probably numerous.

First, not all immunized mothers will have sufficiently potent antibodies to produce clinical symptoms in their offspring.

Second, a certain proportion of these mothers will have Rh negative, unaffected children.

Third, the pigmented skin makes the diagnosis of icterus and anemia very difficult.

Fourth, in our institution Negro multiparas usually stay in the hospital for only twenty-four to forty-eight hours after delivery, which will remove their offspring from clinical observation during the time symptoms may develop (Cases 1 and 2).

Fifth many stillborn infants are not autopsied, and erythroblastosis is probably undiagnosed

Sixth, because marked jaundice is unusual in our group and severe anemia rather common, many cases may have been diagnosed in the past as anemia of the newborn

Whether or not the disease is actually less severe in Negroes cannot be ascertained from this short series

#### SUMMARY

On the basis of a study of 3,115 consecutive obstetric clinic patients of which 86 per cent were Negroes presumptive evidence is submitted that Rh negative Negroes are sensitized at the same rate as Caucasians. The total number of sensitized patients is shown to be directly proportional to the frequency of the Rh<sub>0</sub> negative reaction in a given population. The incidence of affected Negro children in our series was about 1/900 pregnancies. Reasons are suggested for the failure to diagnose erythroblastosis in Negroes at the expected rate.

We wish to thank Dr H H Ware, Jr Professor of Obstetrics, and Dr Lee E Sutton, Jr Professor of Pediatrics, for their kind permission to use the cases cited.

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## NEBULIZED PYRIBENZAMINE IN NASAL AND BRONCHIAL ALLERGY

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THE usefulness of the antihistamine drugs in alleviating the symptoms of nasal allergy is now well established. It is the general experience, however, that many patients fail to be relieved and certain symptoms, such as blocking of the nasal passages, are particularly resistant to these drugs. Among possible explanations for the failure to achieve the desired effect by oral therapy is that an insufficient concentration of the compound reaches the nasal tissues. The toxicity of the present series of antihistamine substances limits the amount which may be given orally to produce a higher nasal concentration. Since in earlier investigations we demonstrated<sup>1, 2</sup> that small quantities of the antihistamine compounds when applied to the skin would inhibit the histamine and allergic reaction, it was considered likely that the topical use of these drugs would be effective in skin<sup>3</sup> and respiratory allergy.

In 1945 we began to experiment with nasal drops of antihistamine drugs. We found that a concentration of Pyribenzamine as low as 0.5 per cent was irritating to the nose and particularly the pharynx of many patients. Recent reports<sup>4, 5</sup> claimed favorable results with such drops. The ordinary nasal spray was also objectionable for similar reasons, although both techniques were helpful in some instances. We finally adopted the technique of administering a nebulized solution from a nebulizer (DeVilbiss No. 40) with an attached nasal piece. This provides an aerosolized mist which is nonirritating and reaches the nasal and pharyngeal tissues most readily.

A 2 per cent buffered solution of Pyribenzamine was found the most generally useful concentration. These topical treatments were not employed routinely for the relief of seasonal hay fever or allergic rhinitis, but were confined to those patients who either were not getting sufficient results from oral antihistamine therapy or who could not tolerate the latter. A number of these patients owed their resistant nasal obstruction to the repeated use of topical vasoconstrictors. Although at times the effect of this nebulization was immediately noticeable, more commonly it was not apparent until later, and particularly after several treatments had been given. In other words, the action of this therapy in these severe cases of nasal congestion is more of a preventive nature and requires repeated administration.

A nebulized mist of 2 per cent Pyribenzamine, released by about half a dozen squeezings of the bulb of a DeVilbiss No. 40 nebulizer, and repeated every two or three hours, produced benefit in twenty-seven out of thirty-four patients with the type of nasal congestion described. This relief had not been obtained in the same patients with the oral use of Pyribenzamine or other

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antihistamine compounds. Of two patients who failed to respond to a 2 per cent solution one responded to a 5 per cent concentration. It should be emphasized that the effect of vasoconstricting drops is more marked and more dramatic than this therapy in relieving nasal obstruction. However the marked congestion which frequently follows the shrinking effect of the repeated use of potent vasoconstrictors is a penalty which frequently is responsible for the continuation of the nasal blocking. The topical antihistamine therapy does not produce this deleterious effect and for that reason is preferable in these difficult cases.

In bronchial allergy the antihistamine drugs orally are much less effective than in nasal allergy. Acute or severe asthma is almost never relieved by these drugs while moderate wheezing may be helped. In spasmodic cough due to bronchial allergy without dyspnea these compounds orally are effective in about one third of the patients. From our experience with topical treatment in these cases we have the feeling that here too the major difficulty probably is the presence of a greater amount of a chemical mediator than can be combated by oral administration of antihistamine drugs. We have employed aerosols of various antihistamine drugs using varying strengths of solutions and various methods of application. Here we wish to record briefly only the results with Pyribenzamine solution as nebulized with the ordinary hand nebulizer or oxygen pressure.

Out of fifty seven patients with asthma, most of whom did not respond to Pyribenzamine by mouth ten obtained effective relief and thirteen had slight relief with a 2 per cent Pyribenzamine aerosol. It should be emphasized that the vast majority of this group were ambulatory patients appearing at the office and presenting a moderate rather than severe asthmatic state. A 1 per cent aerosol was effective in three out of eight patients while a 5 per cent aerosol was effective in five out of nine patients. The spasmodic cough without dyspnea appeared to respond better than the true asthma. Twelve out of sixteen patients reacted favorably. In most instances this type of therapy was less effective than aerosol therapy with epinephrine or isopropyl epinephrine. In several cases of cough the antihistamine aerosol gave better results than these bronchodilators. The main advantage of the use of the antihistamine drugs by aerosol however rests on the much lower toxicity of these solutions and their consequent safety in frequent use as compared with the other aerosols and the antihistamine drugs orally.

#### SUMMARY

The results of the application of aerosolized 2 per cent Pyribenzamine solutions to the nose and bronchi indicate that this is a useful adjunct method in the treatment of selected cases of nasal and bronchial allergy.

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## THE ADAPTABILITY OF MICE TO THE LABORATORY DIAGNOSIS OF TUBERCULOSIS

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THE mouse is ordinarily considered to be resistant to induced tuberculous infection. In reviewing the literature one is at once impressed by the lack of detailed information regarding experimental tuberculosis in mice. Moreover reports by the various investigators reveal a wide divergence of opinion relative to the existence of any reliable and constant characterization of the infection in these animals.<sup>1-10</sup>

Recent studies have stimulated interest in the possibility of using mice for the evaluation of antituberculosis agents<sup>11-15</sup> and for the identification of virulent acid fast bacilli. The latter investigations conducted by Dubos and associates,<sup>16-19</sup> have shown that pigmented (line 1 dba and C 57) strains of mice appeared to be more susceptible to tuberculous infection than other strains examined and, moreover, young cultures grown diffusely in certain liquid media were found to be extremely infective for the mice. It was observed too that deficient (corn meal) diets<sup>20</sup> and also concurrent infection with pneumotropic viruses<sup>21</sup> appeared to accelerate the course of pulmonary tuberculosis in mice.

In light of this recent work the Tuberculosis Evaluation Laboratory has undertaken a series of experiments designed to evaluate the reliability of mouse inoculation tests with special emphasis upon their usefulness in the routine laboratory diagnosis of tuberculosis.

### MATERIALS AND METHODS

*Mice*—Mice of the C57 black and dba strains<sup>16</sup> were obtained from Rockland Farms and at the time of inoculation varied in age from 2 to 11 weeks. Immediately after inoculation the mice were placed in metal cages with wood shavings and wire mesh tops in most instances four or six animals per cage. In the preliminary experiment to be reported and for general maintenance the mice were fed regularly a diet of mouse pellets† and water, but in all subsequent experimental work corn meal‡ was substituted at the time of inoculation.

*Cultures*—For preliminary work in the development of techniques, standard strains§ of human (H37 Rv), bovine (Ravenel) and avian (Kirchberg) tubercle bacilli were used. These stock cultures were maintained in a Tween albumin medium|| patterned after the liquid medium of Dubos<sup>16</sup> in which, after six to seven days, growth appeared as a diffuse, fairly homogeneous suspension containing 0.2 to 0.3 mg. of bacilli (dry weight) per milliliter of medium. Saprophytic acid fast bacilli were used in certain of the experiments as

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‡Rockland Mouse Diet from Rockland Farms, New City, N. Y.

§Obtained from the National Tuberculosis Association Standard Culture Depot at Trudeau, Saranac, N. Y.

||Generously supplied by Difco Laboratories, Detroit, Mich.

controls, and these consisted of organisms recently isolated in this laboratory from sputum specimens. They were avirulent for the guinea pig. The timothy bacillus (*Mycobacterium phlei*) was not used in mouse infection tests involving the liquid medium, since observations here had shown it to grow in a nonacid fast form. A similar observation has previously been reported by Dubos and co-workers<sup>19</sup> in regard to another avirulent strain (H37 Ra). One BCG (Holm) strain was also used, as well as several organisms recently isolated on modified Lowenstein's medium from sputa and gastric washings.

*Inoculation and Autopsy*—Cultures grown diffusely in the liquid medium were inoculated into mice in 0.05 ml. volumes, either directly or diluted to the desired dosage with sterile saline. The modes of infection utilized were (a) intravenously in the tail vein or (b) intracerebrally in the left hemisphere while the animal was under ether anesthesia. Growth from the surface of Lowenstein slants was prepared for mouse inoculation by grinding a weighed sample in a sterile mortar with sterile saline in such a manner that the desired dosage was suspended in 0.05 milliliter. Autopsies were performed on all animals dying during the experimental period (these deaths were attributable, in some part, to intercurrent infections) as well as on all survivors, the latter being sacrificed at the conclusion of the experiments, usually one to four weeks after infection. Evaluations of virulence were based upon the ability of the organisms to proliferate and invade spleen, lung, liver and, in the case of intracerebral inoculations, brain tissue. For purposes of these evaluations, impression smears were prepared from the organs listed and stained by the Ziehl-Neelsen technique, special care being taken, in the case of brain tissue, to prepare smears from the cerebral hemisphere opposite the site of inoculation so that some measure of the degree of spread of the infection within the brain would be available. Arbitrarily, three lengths of each smear were examined microscopically and a record was kept of the total number of acid fast bacilli seen. To express more graphically the extent of the bacterial invasion, the following microscopy code was adopted, based upon the total bacterial count:

1 - 20	= +
21 - 50	= ++
51 - 100	= +++
101 or more	= ++++

Gross lung lesions were designated as being either present (+) or absent (-).

In all instances in which the virulence of the organisms had not been determined previously, guinea pig controls were inoculated simultaneously in the right thigh by the subcutaneous route. The extent of infection was recorded in the following manner, according to the distribution of gross lesions noted at the time of autopsy and confirmed by the demonstration of acid fast bacilli microscopically:

Inguinal lymph node	= Tub 1
All lymph nodes	= Tub 2
Lymph nodes + spleen	= Tub 3
Lymph nodes + spleen + liver	= Tub 4
Lymph nodes + spleen + liver + lungs	= Tub 5

*Experiment 1*—The first experiment was set up with the purpose of deriving a standard technique of mouse inoculation and autopsy for use in subsequent experimental work.

Twenty-four dba mice, 10 to 11 weeks of age, were used, half of which were inoculated intravenously and half intracerebrally. Within each series of twelve mice, six received 0.01 mg. and six, 0.0001 mg. of an H37 Rv culture grown in the Tween albumin medium and injected in a total volume of 0.05 milliliter. In this preliminary experiment it was reasoned that it might be of some value for future comparisons with mice transferred to the deficient diet, to maintain the present animals on regular mouse pellets. At intervals of one, two, and four weeks, two mice in each series of six were killed and autopsied. The lungs of all animals were examined carefully for gross lesions, and at the same time im-



pression smears of lung tissue were stained and examined for the presence of acid fast bacilli. Supplementary smears were prepared of brain tissue in the case of those mice inoculated intracerebrally and of spleen and liver in those inoculated intravenously.

**Results of Experiment 1** Results presented in Table I reveal that bacilli inoculated intravenously in a dose of 0.01 mg can be recovered in lung liver and spleen smears as early as one week after inoculation. Lung smears however, appeared to be inferior from the standpoint of the number of organisms that could be demonstrated early (one to two weeks) in the disease. With the smaller dose (0.0001 mg) there was noted a marked superiority for spleen smears the only organ in which bacilli could be detected in one week. This superiority was present fairly consistently through the entire experiment.

TABLE I RESULTS OF THE INFECTION OF DBA MICE WITH VIRULENT (H37 Rv) TUBERCLE BACILLI BY THE INTRAVENOUS ROUTE

DOSE (MG)	NUMBER OF MICE	AUTOPSY FINDINGS*											
		1 WK				2 WK				4 WK			
		LUNG	SPLEEN	LIVER	GROSS LUNG LESIONS	LUNG	SPLEEN	LIVER	GROSS LUNG LESIONS	LUNG	SPLEEN	LIVER	GROSS LUNG LESIONS
0.01	6	+	++++	++++	-	+	+++	+++	-	+++	+++	+++	-
		+	++++	++++	-	+	+++	+++	-	+++	+++	+++	-
0.0001	6	-	+	-	-	+	+	+	-	+	+++	+	+
		-	+	-	-	-	+++	+	-	+	+++	+	+

\*For explanation of symbols see text

The results of the intracerebral inoculations are summarized in Table II. As in the case of infection by the intravenous route, the initial time of appearance of pulmonary involvement was in fairly close relationship to the size of the inoculum. However, invasion of the cerebral hemisphere opposite the site of inoculation was detectable in the first week regardless of the dosage.

It will be noted that in none of the mice could macroscopic lung lesions be demonstrated nor were there any deaths as a result of the tuberculous infection.

On the basis of results obtained in this preliminary study the intracerebral route of inoculation was adopted in all subsequent experiments since by this method pulmonary involvement was shown to take essentially the course observed after infection by the intravenous route. Moreover, the former technique was preferred because of the relative ease and rapidity with which it can be performed.

TABLE II RESULTS OF THE INFECTION OF DBA MICE WITH VIRULENT (H37 Rv) TUBERCLE BACILLI BY THE INTRACEREBRAL ROUTE

DOSE (MG)	NUMBER OF MICE	AUTOPSY FINDINGS*								
		1 WK			2 WK			4 WK		
		BRAIN	LUNG	GROSS LUNG LESIONS	BRAIN	LUNG	GROSS LUNG LESIONS	BRAIN	LUNG	GROSS LUNG LESIONS
0.01	6	++++	+	-	++++	+	-	+++	+++	-
		++++	+	-	++++	++	-	+	+++	-
0.0001	6	++	-	-	-	+	-	++	-	-
		+++	-	-	+	+	-	+	-	-

\*For explanation of symbols see text.

†One mouse died in twenty-four hours and was eaten by survivors so that no tissue examination was possible.

*Experiment 2*—In this experiment an attempt was made to determine (a) whether tubercle bacilli of all three types are virulent for the mouse, and (b) whether there is any perceptible difference between the three types in respect to their mouse pathogenicity. Young (7 day old) cultures growing diffusely in the Tween albumin medium were used throughout. Strains of tubercle bacilli tested consisted of an avian (Kirchberg), a human (H37 Rv), a bovine (Ravenel), and an acid fast saprophyte recently isolated from sputum and avirulent for the guinea pig. With each strain, six mice of the C57 strain, 4 weeks of age and maintained on a diet of corn meal and water from the time of infection, were inoculated intracerebrally with 0.05 ml of undiluted culture (approximately 0.01 mg dry weight). At one, two, and four week intervals some of the animals were sacrificed and autopsied, at which time the lungs were examined for evidence of gross lesions. Impression smears were prepared of brain, spleen, and lung tissue. Five of the twenty four mice died during the experimental period.

*Results of Experiment 2* As indicated in Table III, all three virulent types produced much the same picture on autopsy. As early as seven days there were signs of marked multiplication of tubercle bacilli in the brain tissue and an invasion of the spleen. In two weeks bacilli were present in large numbers in the lung smears of all animals examined, but only in those inoculated with the bovine strain were there grossly visible lung lesions. These lesions appeared first in those mice killed at the end of two weeks. In none of the six mice inocu-

TABLE III RESPONSE OF C57 MICE TO INOCULATION WITH VIRULENT (BOVINE, HUMAN, AVIAN) AND SAPROPHYTIC ACID FAST BACILLI BY THE INTRACEREBRAL ROUTE.

CULTURE	DOSE (μg)	NUMBER OF MICE	AUTOPSY FINDINGS*				
			DAYS UNTIL AUTOPSY†	BRAIN	SPLEEN	LUNGS	GROSS LUNG LESIONS
Avian (Kirchberg)	0.01	6	K 7	++++	++++	—	—
			K 7	++++	++++	—	—
			D 7	++++	++++	+	—
			K 14	++++	++++	++	—
			K 29	++++	++++	++++	—
			K 29	++++	++++	++++	—
Human (H37 Rv)	0.01	6	K 7	++++	++++	—	—
			K 7	++++	++++	—	—
			D 14	+++	++++	++++	—
			K 14	++++	++++	++	—
			D 18	+	++++	++++	—
			K 28	++++	++++	++++	—
Bovine (Ravenel)	0.01	6	K 7	++++	++++	+	—
			K 7	++++	++++	—	—
			K 14	++++	++++	++++	+
			K 14	++++	++++	++++	+
			D 19	++++	++++	++++	+
			D 24	++++	++++	++++	+
Saprophyte	0.01	6	K 7	+	—	—	—
			K 7	++	—	—	—
			K 14	—	—	—	—
			K 14	+	—	—	—
			K 28	+	—	—	—
			K 28	—	—	—	—

\*For explanation of symbols see text  
 †D=death K=killed the numeral indicates the number of days after inoculation

lated with the saprophyte was it possible to recover acid fast bacilli by smear from the spleen or lung and only an occasional organism was observed in the brain tissue

The results presented in Table III reveal, then, that the C57 mice were susceptible to the three types of virulent tubercle bacilli inoculated intracerebrally. Any conclusion, however, regarding the adaptability of the mice to the type determination of tubercle bacilli could hardly be justified on the basis of the present findings. There were observable differences from the standpoint of certain criteria (deaths, gross lung lesions, relative numbers of bacilli and their time of appearance) but these were not thought to be significant enough for type differentiation.

*Experiment 5*—In the foregoing experiments the results appeared to be encouraging enough to warrant further investigations particularly in respect to a search for a practical application of mouse infection tests in the routine laboratory diagnosis of tuberculosis.

Most laboratories today utilize the classical solid egg media, or modifications of them, in the primary isolation of tubercle bacilli from pathologic material. In order to simulate routine conditions all cultures tested in the experiment to be described were grown on a modified Lowenstein medium as recommended by Holm.<sup>21</sup> In all, twenty-five strains of acid fast bacilli were studied and these included twenty primary isolations from sputum specimens and one from a gastric washing. For purposes of control cultures of standard human (H37 Rv) bovine (Ravenel), saprophytic (timothy), and attenuated mammalian (BCG, Holm) organisms were also included. Mice of the C57 and dba strains (7 to 10 weeks of age) were inoculated intracerebrally with 0.01 mg. of bacilli suspended in 0.05 ml. of sterile saline. Guinea pig controls received the same dosage subcutaneously in the right thigh. The mice were maintained on a diet of corn meal and water from the time of infection, three to four animals of the same age and strain being used for each of the twenty-five cultures.

As shown in Table IV, bacilli cultivated on the solid medium appeared only in the brain tissue of certain of the mice sacrificed at the end of one week. This suggested that these animals might have been killed prematurely and for that reason the period of infection for survivors, was extended to twenty-one days in the remainder of the experiment. At the time of autopsy, impression smears were made of brain, lung and spleen tissue and a record was kept of the presence of macroscopic pulmonary lesions. The guinea pig controls were killed and autopsied at the end of six weeks.

*Results of Experiment 3* Table IV shows that twenty-one of the twenty-five strains studied proved to be virulent for the guinea pig according to the usual criteria. In the mice which had received the corresponding organism in the same dosage, there was, in general, evidence of bacterial proliferation within the brain tissue as well as an invasion of spleen and lung. It will be noted, however, that there occurred some marked inconsistencies at autopsy within groups of animals which had received the same culture in supposedly the same dosage. This phenomenon had been observed, but to a lesser extent in the preceding experiments. Of the factors which might be postulated to have had some bearing on these discrepancies, any one or a combination, of the following might conceivably have been responsible: (a) unconstant dosage because of the presence of clumps of bacilli in both mechanically triturated suspensions and cultures growing diffusely in liquid media,<sup>18</sup> (b) the impossibility, in intracerebral inoculations, of depositing organisms in the same position within the

TABLE IV RELATIVE VIRULENCE, FOR MICE AND GUINEA PIGS, OF CULTURES OF ACID FAST BACILLI

CULTURE*	AGE OF CULTURE (DAYS)	MICE					GUINEA PIGS	
		DAYS UNTIL AUTOPSY†	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY†	AMOUNT OF TUBERCULOSIS‡
H37 Rv	21	K 21	+	-	-	-	D 35	Tub 3
		K 21	++++	+	+++	-		
		K 21	++++	-	++	-		
		K 21	++++	++++	++++	-		
Bovine (Ravenel)	11	D 16	-	-	++	-	K 42	Tub 3
		K 21	++	++++	++	+		
		K 21	-	-	+	-		
		K 21	++++	++++	+	-		
Timothy	6	K 21	-	-	-	-	K 42	-
		K 21	-	-	-	-		
		K 21	-	-	-	-		
		K 21	-	-	-	-		
BCG (Holm)	34	D 13	-	-	-	-	K 42	-
		K 21	-	-	-	-		
		K 21	-	-	-	-		
		K 21	+	-	-	-		
S 1045	36	K 7	-	++	++++	-	K 42	Tub 3
		K 7	-	-	-	-		
		K 21	++++	-	++	-		
		K 21	++++	+	+++	-		
S 1058	35	K 7	+	-	-	-	K 42	Tub 3
		K 7	++++	-	-	-		
		K 21	++	+	+	-		
		K 21	++++	-	+	-		
S 1074	32	K 7	+	-	-	-	K 42	Tub 3
		K 7	++++	-	+	-		
		K 21	++	++++	+++	-		
		K 21	++	++	+	-		
S 604	48	K 7	-	-	-	-	K 42	-
		K 7	-	-	-	-		
		K 21	-	-	-	-		
		K 21	-	-	-	-		
G 55	60	K 7	-	-	-	-	K 42	Tub 3
		D 7	+	-	-	-		
		K 21	++++	+	++++	-		
		K 21	+++	-	+	-		
S 1165	25	K 7	++	-	-	-	K 42	Tub 3
		D 7	++	-	-	-		
		D 15	++++	-	++++	-		
		K 21	++++	-	+	-		
S 701	29	D 1	+	-	-	-	K 42	-
		D 7	+	-	-	-		
		K 21	++	-	-	-		
		K 21	+++	-	-	-		
S 1113	34	K 21	++++	++++	+	-	D 20	Tub 1
		K 21	++++	-	+	-		
		K 21	+++	+	+	-		
		K 21	++++	++++	+++	-		
S 1133	30	K 21	++++	+	+	-	K 42	Tub 3
		K 21	+	-	+	-		
		K 21	+++	-	-	-		
		K 21	+++	-	+	-		

\*S=sputum specimen G=gastric specimen

†D=death K=killed the numeral indicates the number of days after inoculation

‡For explanation see text

TABLE IV—CONT'D

CULTURE	AGE OF CUL- TURE (DAYS)	MICE					GUINEA PIGS	
		DAYS UNTIL AUTOPSY†	BRAIN	LUNG	SILEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY†	AMOUNT OF TUBE- CULOSIS‡
S 1122	34	K 21	++++	++++	+++	-	K 42	Tub ?
		K 21	++++	++++	+++	-		
		K 21	++++	+	++	-		
		K 21	+++	++++	++++	-		
S 1140	36	K 21	++++	++++	++++	+	K 42	Tub 5
		K 21	++++	++++	++++	+		
		K 21	+	+	++	-		
		K 21	-	-	-	-		
S 1140	34	K 21	++++	+	+	-	D 26	Tub 1
		K 21	++++	+	+	-		
		K 21	-	-	+	-		
		K 21	++++	++++	+++	-		
S 1026	29	K 21	++++	+	+++	-	K 42	Tub 3
		K 21	++++	-	+++	-		
		K 21	++++	-	+	-		
		K 21	++++	-	+	-		
S 1226	29	K 21	++	+	++	-	K 42	Tub 1
		K 21	+++	-	+	-		
		K 21	++++	-	++	-		
S 1225	28	K 21	++++	-	-	-	K 42	Tub 1
		K 21	++++	+++	+	-		
		K 21	++++	+	+	-		
S 1270	25	K 21	++++	+	+	-	K 42	Tub 1
		K 21	+	++++	++	+		
		K 21	+	-	-	-		
		K 21	++	+++	+	+		
S 1299	22	K 21	-	+	+	-	K 42	Tub 0
		K 21	+	++	+	-		
		K 21	++++	-	+	-		
		K 21	+	++++	++	-		
S 1297	22	K 21	+	++++	++	+	K 42	Tub 3
		K 21	+	++++	+	+		
		K 21	+	++++	++	+		
		K 21	-	++++	+	+		
S 1168	30	K 21	+	++	+	-	K 42	Tub ?
		K 21	+	-	+++	-		
		K 21	+	++++	+++	+		
S 1253	29	K 21	+++	+	+++	-	K 42	Tub 3
		K 21	+	++++	+	+		
		K 21	++++	++++	+++	+		
		K 21	+	++++	++	-		
S 1250	28	K 21	++	-	-	-	K 42	Tub 1
		K 21	++++	-	-	-		
		K 21	++	+	+	-		
		K 21	-	-	-	-		

brain of every mouse and the relationship this might possibly have to the rate of bacterial spread (c) the usual errors of random sampling encountered in the selection of material for smear and in the choice of fields in microscopic examinations and finally (d) it can be assumed with some degree of certainty that mice even though they be of the same age and strain probably vary considerably in their native susceptibility to tuberculous infection.

From an analysis of the data presented in Table IV it can be concluded nevertheless that in the strains of acid fast bacilli examined and on the basis

of the evaluation procedure established for the determination of pathogenicity in the present study, there was evidence of close correlation between virulence for mice and virulence as measured by classical guinea pig tests

*Experiment 4*—The results presented in the preceding experiments indicated that a combination of rapid, diffuse culture in a liquid medium, plus subsequent mouse inoculation, would reduce considerably the time required for the diagnosis of tuberculosis in the routine laboratory. Experiment 4 was designed to explore this possibility.

A recent report by Foley<sup>23</sup> described the successful use of the Dubos medium for the rapid, primary isolation of tubercle bacilli from various pathologic materials. Special note, however, was made of the fact that the medium supports the growth of a variety of nonacid fast organisms. Of considerable interest, then, was the work of Goldie<sup>24</sup> in which the addition of penicillin (0.5 to 2 units per milliliter) to the Dubos medium was advocated in light of its suppressive effect on the growth of contaminants.

For this experiment a total of nine sputum specimens was selected at random. A direct smear was prepared of each specimen and a 2 ml portion was prepared for culture according to the classical NaOH concentration technique.

With a sterile Pasteur pipette, approximately 0.25 ml of each neutralized (2N HCl) sediment was inoculated into a tube containing 5 ml of the Tween albumin medium, to which had been added penicillin in the amount of 2 units per milliliter. To serve as controls, identical inocula of each sediment were spread evenly over the surface of two tubes of the Lowenstein medium.

After incubation periods of eight, ten, fourteen, fifteen, and twenty one days certain of the Tween albumin cultures were prepared for animal inoculation by first centrifuging 3 ml samples at 3,000 rpm for forty five minutes. The supernatant fluid was discarded and 0.5 ml of sterile saline was added to the sediment. Dba mice, varying in age from 8 to 11 weeks and maintained on a diet of corn meal and water from the time of inoculation, were inoculated intracerebrally with 0.05 ml of the saline suspension, four animals being used for each of the nine cultures. The experimental period was twenty one to twenty six days, at the end of which time all the mice were sacrificed and autopsied according to the technique described in the preceding experiments. Four of the thirty six mice died during the observation period. Guinea pig controls received 0.05 ml of the saline suspensions subcutaneously in the right thigh and were autopsied after an infection period of six weeks.

It is interesting to note that a preliminary examination of the direct smears of the original sputum specimens revealed that five were positive for acid fast bacilli, whereas four were negative.

*Results of Experiment 4* The results are summarized in Table V. It can readily be seen that penicillin in a concentration of 2 units per milliliter in the Tween-albumin medium was toxic to the tubercle bacilli contained in the experimental inocula. These results stand in sharp contrast to those of Goldie previously described, but would appear to coincide well with the investigations of Kirby and Dubos<sup>25</sup> which revealed that small inocula of tubercle bacilli are highly susceptible to concentrations of penicillin as low as 1 unit per milliliter in Tween-albumin medium. Reports of results from other laboratories,<sup>26</sup> where comparative studies have been made, have advised against the routine use of liquid media in the isolation of tubercle bacilli from pathologic material. Such findings bear out the observations of Dubos<sup>27</sup> who, following a recent evaluation of his own medium, concluded that, "It would appear unwise to formulate in final form the composition of media best adapted for diagnostic work until more is known of the growth requirements of the different strains of tubercle bacilli which occur in pathological material."

TABLE V RESPONSE OF dba Mice TO THE INTRACEREBRAL INOCULATION OF PRIMARY SPUTUM CULTURES IN TWELFEN ALBUMIN-PENICILLIN (2 UNITS/ML) MEDIUM SUMMARY OF RESULTS OBTAINED WITH 9 RANDOM SPUTUM SPECIMENS

MICROSCOPY (DIRECT SMEAR)	AUTOPSY FINDINGS		LOWENSTEIN CULTURE CONTROLS (WITHOUT PENICILLIN)
	ANIMALS INOCULATED WITH TWEEEN ALBUMIN-PENICILLIN CULTURES		
	MICE (21-26 DAYS)	GUINEA PIGS (42 DAYS)	
Number positive = 5	-	-	+
Number negative = 4	-	-	-

All five positive cultures were shown to be virulent human type tubercle bacilli by subsequent guinea pig inoculation

Further investigations into the possibility of deriving a more perfect combination of rapid diffuse culture from pathologic material with mouse confirmation await then the formulation of a reliable liquid medium with the ability to initiate growth of minimal inocula of tubercle bacilli quickly and uniformly and of equal importance the ability to eliminate all contaminating microorganisms

*Experiment 5*—The final study in the present series was devoted to an evaluation of the adaptability of mice to the isolation of tubercle bacilli directly from pathologic material. A survey of the literature reveals that investigations of this nature have been neglected. Only recently<sup>18</sup> has there been reported the successful establishment of a tuberculous infection in mice by the injection intracerebrally of 0.01 ml of sputum obtained from tuberculous patients.

In the following experiment two routine sputum specimens (S-2205 and S-2206) were prepared for mouse inoculation according to the procedure previously outlined (Experiment 4). After neutralization with 2N HCl approximately 0.25 ml of the sediment was seeded on each of two Lowenstein slants and the balance was transferred aseptically to an inoculating bottle containing 1 ml of sterile distilled water. Mice of the dba strain 2 to 5 weeks of age were inoculated intracerebrally with 0.05 ml of this suspension, four animals being used for each specimen. All mice were maintained on the deficient diet during the experimental period. The unused portion of each suspension was inoculated subcutaneously into the right groin of a control guinea pig. Infection periods and autopsy techniques for both mice and guinea pigs were essentially those described in the preceding experiments.

As can be seen from the data presented in Table VI these two digested (NaOH) and neutralized (HCl) sputum specimens proved to be extremely toxic to the mice. Only three of the eight animals inoculated survived the twenty one day infection period. It might be worth mentioning that one additional unrecorded sputum sample killed all four mice instantly. In an effort to determine whether this toxicity might be due to some factor present in untreated material the balance of this study was confined to an observation of the effect on the mice of clear etherized urine specimens which ordinarily require no NaOH homogenization. Each specimen was first centrifuged at 3000 rpm for fifteen minutes following which the supernatant fluid was removed. Procedures for culture and for mouse and guinea pig inoculations were identical with those outlined for the sputum specimens. In Table VI these specimen numbers are preceded by the letters BF. This study was terminated when

it was recognized that the untreated urines were similarly toxic to the mice. Of a total of thirty-six animals, twenty (55 per cent) died during the experimental period.

TABLE VI RESULTS OF THE INOCULATION OF ROUTINE CLINICAL SPECIMENS SIMULTANEOUSLY INTO MICE, GUINEA PIGS, AND CULTURE MEDIA, MOUSE DOSAGE = 0.05 ML OF DILUTED (DISTILLED WATER) SEDIMENT INTRACEREBRALLY

SPECI MEN	TYPE OF SPECI MEN	AUTOPSY FINDINGS							CUL TURES
		MICE					GUINEA PIGS		
		NUMBER DEAD/ TOTAL	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY†	AMOUNT TBC‡	
S 2205	Sputum	3/4	0.5+*	0	0	-	K 42	-	-
S 2206	Sputum	2/4	3.0+	0.5+	2.0+	-	K 42	Tub 1	+
BF 265	Urine	3/4	0	0	0		K 42	-	
BF 266	Urine	1/4	0	0	0		K 42	-	-
BF 271	Urine	1/4	0	0	0		K 42	-	-
BF 274	Urine	3/4	0	0	0		K 42	-	-
BF 276	Urine	4/4	0	0	0		K 42	-	
BF 277	Urine	1/4	0	0	0	-	K 42	-	
BF 278	Urine	3/4	0	0	0		K 42	-	-
BF 284	Urine	2/4	0	0	0		K 42	-	-
BF 292	Urine	2/4	0	0	0		K 42	-	

\*In this and subsequent tables this numeral indicates the average of the 0 to +++ ratings of the individual mice. Autopsies were not performed on those mice which died at the time of inoculation.

†K=killed the numeral indicates the number of days after inoculation.

‡For explanation see text.

In light of the fact, however, that in one instance (S 2206) the results following mouse inoculation were confirmed by guinea pig inoculation and culture and in another (S 2205) the results indicated a possible superiority for mouse inoculation had the infection period been extended, it was thought that it might be of some value to attempt to minimize or perhaps eliminate the toxic manifestations by introducing certain changes in the technique. With this in mind a new series of ten random pathologic specimens was prepared for animal inoculation and culture by the NaOH concentration technique. Procedures for animal inoculation, autopsy, and culture were identical to those described with the exception that sterile physiologic saline was substituted for distilled water as a diluent, the volume of the intracerebral inoculum was reduced from 0.05 ml to 0.03 ml, and the infection period for mice was extended to six weeks.

*Results of Experiment 5* The results are summarized in Table VII. It will be noted that two of the specimens (BF-223 and G-180) killed all four mice instantly. As shown in the table, a total, then, of seventeen (42 per cent) mice died during the six-week infection period. This indicated only a slight reduction in the casualty rates in spite of the modified technique and it seems reasonable to assume that this persistently high percentage of premature deaths will markedly limit the usefulness of mice in the isolation of tubercle bacilli directly from pathologic materials by the methods described. Moreover, the failure, in two instances (S-1365 and G-179), of definitive mouse confirmation of the guinea pig and culture results would seem to indicate that the extended infection period was of no particular advantage. It is interesting to note at this point that the pronounced toxicity of clinical specimens for mice recently has been observed in another laboratory.<sup>25</sup>



TABLE VII. RESULTS OF THE INOCULATION OF ROUTINE CLINICAL SPECIMENS SIMULTANEOUSLY INTO MICE GUINEA PIGS AND CULTURE MEDIA. MOUSE DOSAGE = 0.03 ML. OF DILUTED (SALINE) SEDIMENT INTRACEREBRALLY

SPECIMEN	TYPE OF SPECIMEN	AUTOPSY FINDINGS							
		MICE				GUINEA PIGS			
		NUMBER DEAD/TOTAL	BRN	LLC	SLIFEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY	AMOUNT TBC	CULTURES
BF 220	Sputa	2/4	0	0	0	-	K 42	-	-
BF 218	Pleural fluid	1/4	0	0	0	-	K 42	-	-
S 1366	Bronchial washing	1/4	0	0	0	-	K 42	-	-
S 1365	Bronchial washing	2/4	0	0	0.2+	-	K 42	Tub 1	+
G 175	Gastric	1/4	0	0	0	-	K 42	-	-
G 178	Gastric	1/4	0	0	0	-	K 42	-	-
G 179	Gastric	0/4	0	0	0	-	K 42	Tub 3	-
BF 2-4	Urine	1/4	0	0	0	-	K 42	-	-
BF 2-3	Urine	4/4	-	-	-	-	K 42	-	-
L 180	Gastric	4/4	-	-	-	-	K 42	-	-

*Experiment 6*—A final experiment was devised to test the possibility of rendering pathologic materials less toxic for the mice by (a) increasing the volume of saline diluent and (b) washing the sediment. For this purpose six microscopically positive sputum specimens were digested (NaOH) and concentrated and to each neutralized (2N HCl) sediment were added 4 ml of sterile physiologic saline. Half (2 ml) of each sediment was then transferred aseptically to a sterile centrifuge tube and the material centrifuged for fifteen minutes at 3000 r.p.m. After removal of the supernatant fluid the material was resuspended in 2 ml of fresh sterile saline and a portion of each (washed and unwashed) sediment was used for the inoculation of mice guinea pigs and culture media. The intracerebral mouse inoculum was 0.03 ml and the infection period was twenty one days.

The data summarized in Table VIII reveal a sharp reduction in the number of nontuberculous deaths among the mice. Only eleven (22 per cent) animals died during the twenty one day experimental period. All of these deaths occurred within a period of from one to fourteen days after inoculation. However an examination of the findings also reveals that in none of the mice was there evidence of generalized tuberculosis (note the absence of both gross and microscopic lung pathology) although guinea pig results had indicated the presence of virulent tubercle bacilli in all but one specimen (S 976). Moreover it would appear that in general the degree of infection among mice inoculated with washed sediments was less than that in the group receiving unwashed material. It seems probable then that some diminution in the number of bacilli occurred as a result of the dilution and washing techniques employed. This disadvantage although probably slight in the case of specimens containing large numbers of organisms would gain increasing significance in the case of those materials containing only a few bacilli. Any significant number of failures would constitute then, what is probably the most valid objection to this method as a practical means of using mice for the isolation of tubercle bacilli directly from pathologic materials.

A recent study of particular significance which should be considered here is that of Milzer and Levine<sup>29</sup> who, using pigmented strains of mice, reported several successful isolations of tubercle bacilli from clinical specimens (sputa and feces) ten to fifteen days after the intraperitoneal inoculation of suspensions containing gastric mucin, rather than saline, as a diluent. No mention was made of any toxic effect on the mice.

TABLE VIII. RESULTS OF THE INOCULATION OF ROUTINE SPUTUM SPECIMENS SIMULTANEOUSLY INTO MICE, GUINEA PIGS, AND CULTURE MEDIA, COMPARISON OF UNWASHED (A) AND WASHED (B) SEDIMENTS

SPECI MEN	SEDI MENT	AUTOPSY FINDINGS							CULTURE
		MICE					GUINEA PIGS		
		NUMBER DEAD/ TOTAL	BRAIN	LUNG	SPLEEN	GROSS LUNG LESIONS	DAYS UNTIL AUTOPSY	AMOUNT TBC	
S 676	a	0/4	4 0+	0	2 5+	-	K 42	Tub 5	+
	b	2/4	1 5+	0	1 0+	-	K 42	Tub 4	+
S 680	a	1/4	3 3+	0	2 7+	-	K 42	Tub 5	+
	b	1/4	3 0+	0	3 0+	-	K 42	Tub 5	+
S 681	a	1/4	1 5+	0	1 0+	-	K 42	Tub 5	+
	b	0/4	1 7+	0	0 7+	-	K 42	Tub 5	+
S 912	a	0/4	1 0+	0	0	-	K 42	Tub 3	+
	b	1/4	0	0	0	-	K 42	Tub 3	+
S 961	a	1/4	1 2+	0	0 5+	-	K 42	Tub 5	+
	b	2/4	0 2+	0	0	-	K 42	Tub 3	+
S 976	a	0/4	0 5+	0	0	-	K 42	-	+
	b	2/4	0	0	0	-	K 42	-	+

#### DISCUSSION

From time to time in the past, and with renewed vigor in recent years, the possibility has been suggested that mice might be found useful in certain types of investigations in the field of tuberculosis. Only one of these types of investigation has been considered here, namely, the detection of virulent tubercle bacilli in pathologic materials. It should be emphasized that sporadic attempts to develop techniques whereby the mouse might conceivably supplant the guinea pig as a criterion of virulence have been made ever since the discovery of the tubercle bacillus by Koch in 1882. It is probably because marked discrepancies were revealed by the various workers that nothing of a practical nature has materialized. In addition, certain other objections have been raised against the mouse as a test animal in the diagnosis of tuberculosis. It has been suggested,<sup>9</sup> for example, that the failure of the mouse to develop tuberculin sensitivity renders the detection of spontaneous tuberculous infections in mice impossible. This disadvantage gains increasing significance in light of the work of Glover,<sup>10</sup> who determined that the inhalation, by mice, of as few as fifty to one hundred virulent tubercle bacilli resulted in the establishment of recognizable tuberculous foci. Guinea pigs, on the other hand, can and should be tuberculin tested before use to rule out the presence of spontaneous disease.

Another serious criticism of the general adaptation of mice for tuberculosis diagnostic work results from the finding by certain investigators<sup>7</sup> that among human strains of tubercle bacilli there are encountered some which are *not* of equal virulence for both guinea pig and mouse. Until it can be demonstrated,

through extensive and painstaking investigation, that there exists an exact parallel between virulence for mice and virulence for man, it is doubtful that mouse infection tests will gain general recognition. The objection might be raised, too, from observations made in this laboratory and in others,<sup>11</sup> that the inoculation of mice with virulent tubercle bacilli is not always followed by a constant and predictable degree of infection among animals receiving identical dosages of the same organism.

It was the purpose of the present study to (a) verify some of the more practical laboratory findings concerned with mouse infection tests, and (b) to establish criteria for evaluating the reliability of such tests, some of which will be discussed here briefly.

That different strains of mice vary markedly in their susceptibility to induced tuberculosis has been observed by Dubos and co-workers<sup>12</sup> and it was on the basis of their findings that pigmented (C57 and dba) rather than white animals were used exclusively in the experimental work reported herein. As noted by the Rockefeller group<sup>13</sup> however, "It is probable that unrecognized variations in the culture, the season of the year and other uncontrolled factors have an appreciable influence on the course and outcome of the experimental infection." In addition it is probable that individual mice of the same strain differ from others in the group as a result of certain as yet unknown factors. These might make mandatory a more thorough investigation of identical strains of mice obtained from different breeders. Pending the completion of such studies it would perhaps be wise for each laboratory to maintain a constant and reliable source of mice to eliminate the necessity of re-evaluating new and untried groups of animals.

Another source of variation is the enhancing effect, on tuberculous infections, of a deficient (essentially corn meal and water) diet which has been demonstrated for albino and pigmented mice<sup>14</sup> as well as rats.<sup>15</sup> This experimental evidence was convincing enough to warrant the inclusion of such diets in studies carried on by this laboratory in order to increase susceptibility of our mice to the test infections.

With respect to the mode of inoculation the intracerebral rather than the intravenous technique was preferred here because of its simplicity and the relative ease with which it can be mastered by the average technician.

The size and time relationships of dosage, all too frequently lost sight of in tuberculosis diagnostic work should be given careful consideration in mouse infection tests. On the basis of investigations conducted here and in other laboratories, a dosage of 0.01 mg. has been arbitrarily adopted for use with cultures of tubercle bacilli. It was felt that the ideal dosage should be one which facilitates an early (one to three weeks) detection of proliferation in the case of pathogens without being so overwhelming as to produce false positives in the case of saprophytes, a phenomenon which though yet to be demonstrated with mice is not unknown in guinea pigs. It can be seen from the experimental results presented that tubercle bacilli can be detected in the various organs by smear even though gross evidence of their presence is lacking. This facilitates

early diagnosis. However, gross pulmonary lesions should be examined microscopically in every instance in order to exclude confusion due to spontaneous disease closely simulating tuberculosis. Such conditions have been demonstrated in rats.<sup>32</sup> In addition, in two instances in our laboratory pulmonary lesions indistinguishable macroscopically from tubercles have been observed in mice at autopsy in which a diligent search failed to reveal the presence of acid fast bacilli.

Finally, in regard to the minimum number of animals indicated for valid results, it is recommended that at least four mice be inoculated with each culture to compensate not only for inconstant degrees of tuberculous infection among the animals but also for deaths due to intercurrent infections.

#### SUMMARY

The susceptibility of pigmented strains of mice (dba and C57) to tuberculosis induced by the intracerebral and intravenous inoculation of virulent human tubercle bacilli has been confirmed.

In these studies, tubercle bacilli of the human, bovine, and avian types were of similar virulence for mice following inoculation by the intracerebral route. An acid-fast saprophyte failed to produce a progressive disease.

In a comparative study involving a series of twenty-five strains of acid-fast bacilli there appeared to be a high degree of correlation between virulence for mice and virulence as measured by classical guinea pig tests.

Preliminary studies indicated that a combination of rapid, diffuse growth in a liquid medium of the Dubos type plus subsequent mouse inoculation would reduce considerably the time required for the laboratory diagnosis of tuberculosis. Penicillin, added to a Tween-albumin medium in a concentration of 2 units per milliliter with the purpose of suppressing the growth of contaminants, proved to be toxic to the tubercle bacilli contained in experimental inocula consisting of digested and neutralized sputum specimens.

Both treated (NaOH) and untreated routine clinical specimens were shown to be extremely toxic when inoculated (intracerebrally) directly into mice in an attempt to recover tubercle bacilli, therefore the routine use of mice as a primary diagnostic procedure is not practical by the methods described.

The theoretical and practical aspects of mouse infection tests are discussed in some detail.

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## THE SEROLOGIC RELATIONSHIP OF FUNGUS ANTIGENS

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THE reported concurrence of pulmonary calcification and skin sensitivity to histoplasmin<sup>1-5</sup> has led to the suggestion that infection with *Histoplasma capsulatum* is frequently nonfatal. This depends on the theory that sensitivity to histoplasmin results from a past or present infection with *H. capsulatum*. Emmons and co-workers<sup>6, 7</sup> have demonstrated, however, that a positive skin reaction to histoplasmin may appear in animals experimentally infected with either *H. capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, or *Candida albicans*. Howell<sup>8</sup> demonstrated by skin tests the extensive cross reaction between histoplasmin and blastomycin in guinea pigs infected with *H. capsulatum* and *B. dermatitidis*.

Several workers started serologic investigations with the hope that the information therefrom would aid in the diagnosis of a mild or severe form of histoplasmosis. I<sup>9</sup> described the use of the whole yeastlike cell as a complement fixation antigen, and found it more specific than histoplasmin. Tenenbein and Howell<sup>10</sup> found that both histoplasmin and blastomycin, when used as complement fixation antigens, produced positive reactions in the presence of sera from animals experimentally infected with *B. dermatitidis* or *H. capsulatum*. Fixation was stronger with the homologous than with the heterologous antigen. Saslaw and Campbell,<sup>11</sup> using the sera of rabbits hyperimmunized with heat-killed antigens, found cross reactions between the yeastlike phase of *H. capsulatum* as antigen and anti-*B. dermatitidis* serum, but more especially between the yeastlike phase of *B. dermatitidis* and anti-*H. capsulatum* serum.

With the indication that antigens from the yeastlike phase appeared to be more specific than those from the mycelium, but with little knowledge of the extent of cross reactions between fungus antigens and of the identity of factors in the various antigens, further study of the serologic relationship between fungus antigens seemed desirable. Accordingly, experiments were initiated to determine by complement fixation methods the specificity of and relationships between antigens from *H. capsulatum*, *B. dermatitidis*, *C. immitis*, *C. albicans*, *Aspergillus fumigatus*, and *Mucor corymbifer*. The first four fungi were chosen for study since Emmons and associates<sup>6, 7</sup> had already established their positive immunologic relationship, in that guinea pigs infected with one or them were capable of showing positive skin reactions to histoplasmin. The last two were included for study because, although pathogenic, they had not been shown as having any relationship to *H. capsulatum* by skin test and because they were so decidedly different morphologically from *H. capsulatum*.

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## MATERIALS AND METHODS

**Complement Fixation Test**—The complement fixation test, originally described by Bengston<sup>12</sup> was similar to the one reported in a previous paper.<sup>9</sup>

**Fungi**—One strain of each of the six fungi was employed in these studies. Strain 6521 of *H. capsulatum* originally was isolated from a dog<sup>13</sup>, strain 6046 of *B. dermatitidis* from a human patient in Alabama, strain 6210 of *C. immitis* from a human patient in Arizona, strain 3148 of *C. albicans* from a human patient with endocarditis, strain 5225 of *A. fumigatus* from a penguin, and strain 217 of *M. corymbifer* from a pea. The antigens from *H. capsulatum*, *B. dermatitidis* and *C. albicans* were whole yeastlike cells, which had been killed by suspension in 0.5 per cent formalin for eighteen hours, washed in saline, centrifuged, and then stored in a concentrated form at 2 to 5°C in 1:10,000 Merthiolate. Since the sporangial phase of *C. immitis* has not been grown extensively in culture, the broth filtrate from mycelial growth (i.e., coccidioidin) was used as the antigen. Washed spores served as the antigens for *A. fumigatus* and *M. corymbifer*. *H. capsulatum* was grown in the yeastlike phase in a semifluid peptone medium<sup>14</sup>, *B. dermatitidis* in a semifluid medium containing a mixture of amino acids plus serine<sup>15</sup>, *C. albicans* in Sabouraud's dextrose broth, and *A. fumigatus* and *M. corymbifer* on Sabouraud's dextrose agar. The antigens were assayed against homologous immune sera at irregular intervals to check their binding power and anti-complementary action.

**Antisera**—Forty adult albino rabbits were inoculated (a) intravenously with living yeastlike cells (e.g., ten rabbits with *H. capsulatum* and ten with *B. dermatitidis*), (b) intraperitoneally with a suspension of live conidia (e.g., fourteen rabbits with *C. immitis*) or (c) intravenously with increasing dosages of formalin-killed yeast cells (e.g., six rabbits with *C. albicans*). The rabbits were not inoculated with live cells of *C. albicans* because infection with this agent produced rapid death in such animals. The groups of rabbits that were inoculated with one of the live strains were bled once a week thereafter until the termination of the experiment. The sera that were used for the absorption studies were pools from these and other similar bleedings. Only those sera with positive titers with the homologous antigens were included in these pools. Attempts to produce hyperimmune anti-*A. fumigatus* and anti-*M. corymbifer* sera of significantly high titer were unsuccessful, with the result that these two types of sera were not used in the absorption tests in the present study.

## EXPERIMENTS AND RESULTS

Individual sera from thirty-four rabbits experimentally infected with *B. dermatitidis*, *C. immitis* or *H. capsulatum* and bled once a week were titrated against their homologous antigens. Those sera which fixed complement in the presence of the homologous antigen were tested against the three heterologous antigens (Figs 1 to 3). The *B. dermatitidis* antigen induced the formation of antisera only 8 per cent of which (seven of ninety-two sera) fixed complement with the heterologous *H. capsulatum* antigen and none of which reacted with the *C. albicans* or *C. immitis* antigens. The titers with the *H. capsulatum* antigen against the anti-*B. dermatitidis* serum were noticeably lower than those with the *B. dermatitidis* antigen (Fig 1). However, *B. dermatitidis* was weaker as an antibody-producing antigen than the other antigens, as evidenced by the fact that its antisera had much lower titers than the other antisera, when each was tested against its homologous antigen (Figs 1 to 3). The *H. capsulatum* antigen induced antisera which fixed complement in equal quantity with the homologous and the *B. dermatitidis* antigens, although the antibody titers with the latter were slightly lower (Fig 2). None of the anti-*H. capsulatum* sera reacted with the antigens from *C. albicans* and *C. immitis*. The

This antigen was obtained from Dr C. E. Smith, Stanford University School of Medicine, San Francisco, Calif.

*C. immittis*-infected animals produced antisera which reacted not only with the homologous antigen but 63 per cent of which also reacted with the *B. dermatitidis* antigen and 6 per cent with the *H. capsulatum* antigen. The antibody titers with *B. dermatitidis* and especially with *H. capsulatum* antigens were

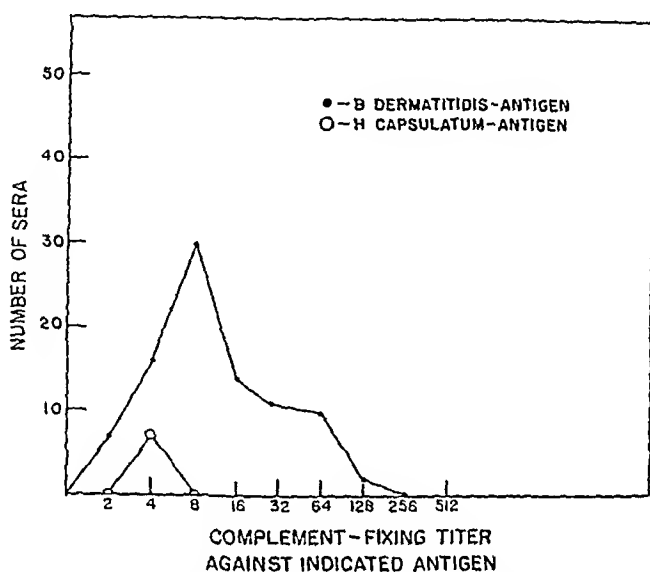


Fig. 1—Reactivity of anti *B. dermatitidis* sera with homologous and heterologous antigens. Note: No positive titers were produced with *C. immittis* and *C. albicans* antigens.

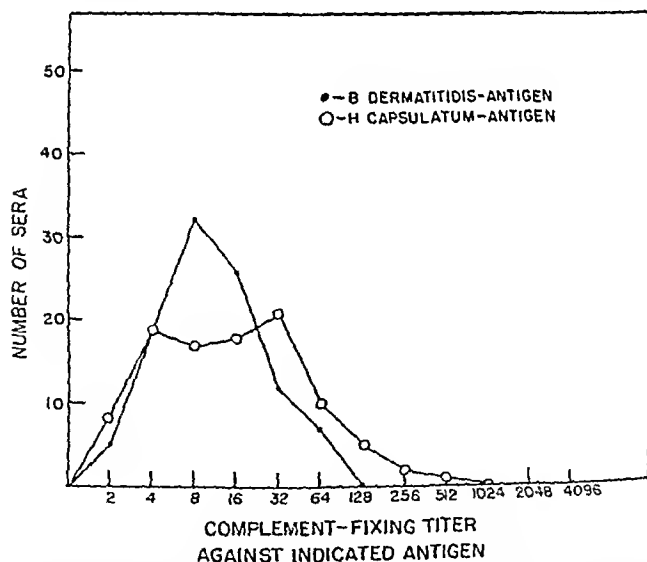


Fig. 2—Reactivity of anti-*H. capsulatum* sera with homologous and heterologous antigens. Note: No positive titers were obtained with *C. albicans* and *C. immittis* antigens.

much lower than with the homologous antigen. No fixation occurred in the presence of the *C. albicans* antigen. The pooled serum from the rabbits hyperimmunized with the *C. albicans* antigen had an antibody titer of 1:256 with the homologous antigen, of 1:16 with the *H. capsulatum* antigen, and of 1:2 with the *B. dermatitidis* antigen. No titer was obtained with the *C. immittis* antigen.



Thus *B dermatitidis* antigen may be considered as forming the most specific antibody (of the four tested) although its complement fixing antigen was the least specific. *H capsulatum* induced the formation of a slightly less specific antibody than *B dermatitidis* while *C albicans* and *C immitis* produced antisera that cross reacted to the greatest extent with heterologous antigens. It may be noted here that the serum titers were relatively low even with the homologous antigens a characteristic common to most fungus antisera.

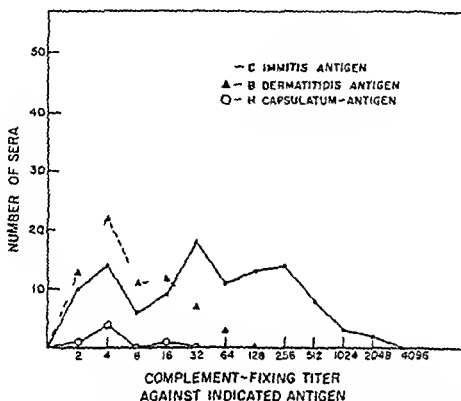


Fig. 3—Reactivity of anti *C immitis* sera with homologous and heterologous antigens. Note: No positive titers were obtained with the *C albicans* antigens.

Further information on the interrelationship of the fungal antigens was sought in studies involving absorption of antisera with both homologous and heterologous antigens. Pooled rabbit antisera were absorbed with an antigen from one of six fungal species namely *H capsulatum*, *B dermatitidis*, *C albicans*, *C immitis*, *A fumigatus* and *M corymbifer*. The antigens from the first three species were whole yeastlike cells killed by 0.5 per cent formalin. In the case of *C immitis* the antigen used for absorbing antibody was mycelium grown for three weeks at 25° C, killed in 0.5 per cent formalin, washed in saline and ground thoroughly in a ball mill. The antigens from *A fumigatus* and *M corymbifer* were spores washed off agar slants, rewashed in saline, killed in 0.5 per cent formalin, then washed and resuspended in saline. The serum was absorbed with homologous antigen until either no serum titer remained or additional absorption produced no change in titer. Twice the amount of antigen used in the foregoing experiments was employed in the absorption of heterologous antisera. In the tests involving *A fumigatus* and *M corymbifer* the sera were absorbed and reabsorbed until they started to become anticomplementary. The experiments were repeated three times with different sera with similar results obtained except where indicated.

When the anti-*B dermatitidis* serum was absorbed with its homologous antigen (Table I), a similar decrease in titer of the serum was shown with both the *B dermatitidis* and the *H capsulatum* complement-fixing antigens. This result indicates that the *B dermatitidis* antigen induces the formation of one antibody or one group of antibodies which is completely responsible for the serologic reaction between anti-*B dermatitidis* serum and *H capsulatum* antigen. Absorption with the *C albicans*, *C immitis*, or *H capsulatum* antigen resulted in partial removal of reactivity with the homologous *B dermatitidis*

TABLE I INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION TITERS OF RABBIT ANTISERA

ANTIGEN	UNABSORBED	ANTI- <i>B DERMATITIDIS</i> SERUM					
		ABSORBED WITH					
		<i>B DERMATITIDIS</i>	<i>H CAPSULATUM</i>	<i>C ALBICANS</i>	<i>C IMMITIS</i>	<i>A FUMIGATUS</i>	<i>M CORYMBIFER</i>
<i>B dermatitidis</i>	32	1*	4	1	16	32	16†
<i>H capsulatum</i>	16	1	0	1	0	16	16
<i>C albicans</i>	0‡	—	—	—	—	—	—
<i>C immitis</i>	0	—	—	—	—	—	—

\*Further absorption produced no decrease in titer.

†The figures are the inverse of the maximum serum dilution that completely fixed complement in the presence of 1 units of antigen.

‡Throughout this paper '0' indicates no fixation in a serum dilution of 1:2.

complement fixation antigen and almost complete removal with the *H capsulatum* complement-fixing antigen. The *C albicans* antigen seemed to have relatively strong absorptive qualities for the anti-*B dermatitidis* serum especially when it is noted that this antigen did not fix complement with this serum. Apparently there was not sufficient *C albicans* antigen in the serologic test to produce a reaction in the presence of the heterologous antiserum. Thus, the antibodies induced by the *B dermatitidis* antigen appear to contain a factor or factors which are capable of combining in varying degrees, depending on the species, with the preceding three heterologous antigens. *A fumigatus* and *M corymbifer* absorbed no significant amount of antibody from the anti-*B dermatitidis* serum, and therefore probably have no antigenic similarity with *B dermatitidis*.

Although anti-*C albicans* serum fixed complement in the presence of *B dermatitidis* and *H capsulatum* antigens in low titers, absorption with the homologous *C albicans* antigen removed the cross reactions with these two heterologous antigens (Table II). Thus, the *C albicans* antigen contains a factor that is the same in serologic reaction as the one in *B dermatitidis* and *H capsulatum* antigens that causes fixation of complement in the presence of anti-*C albicans* serum. Absorption with either *B dermatitidis* or *H capsulatum* antigens resulted in the removal of antibody from anti-*C albicans* serum, as shown with the *C albicans*, *B dermatitidis*, or *H capsulatum* complement fixation antigens. When the antiserum was absorbed with *C immitis* antigen, only part of the antibody was removed. These two observations indicate that the

anti *C. albicans* serum has a substance that can react well with *B. dermatitidis* and *H. capsulatum* antigens and to a more limited extent with *C. immitis* antigen. Because of their failure to absorb any antibody from anti *C. albicans* serum, *A. fumigatus* and *M. corymbifer* antigens were considered unrelated to the *C. albicans* antigen.

TABLE II INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION TITERS OF RABBIT ANTISERA

ANTIGEN	ANTI <i>C. ALBICANS</i> SERUM						
	UNABSORBED	ABSORBED WITH					
		<i>B. DERMATITIDIS</i>	<i>H. CAPSULATUM</i>	<i>C. ALBICANS</i>	<i>C. IMMITIS</i>	<i>A. FUMIGATUS</i>	<i>M. CORYMBIFER</i>
<i>C. albicans</i>	256	0	0	0	64	256	256
<i>B. dermatitidis</i>	2	0	12	0	12	12	2
<i>H. capsulatum</i>	16	0	0	0	8	8	16
<i>C. immitis</i>	0	-	-	-	-	-	-

Anti *C. immitis* serum after absorption with its homologous antigen still partly reacted with the *B. dermatitidis* and *H. capsulatum* complement fixation antigens (Table III). Thus, there are nonspecific factors in the anti *C. immitis* serum which react serologically with the *H. capsulatum* and *B. dermatitidis* antigens, but not with the homologous antigen. Absorption with *B. dermatitidis* and *H. capsulatum* antigens resulted in almost complete loss of antibody capable

TABLE III INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION OF RABBIT ANTISERA

ANTIGEN	ANTI <i>C. IMMITIS</i> SERUM						
	UNABSORBED	ABSORBED WITH					
		<i>B. DERMATITIDIS</i>	<i>H. CAPSULATUM</i>	<i>C. ALBICANS</i>	<i>C. IMMITIS</i>	<i>A. FUMIGATUS</i>	<i>M. CORYMBIFER</i>
<i>C. immitis</i>	32	0	0	16	0	32	16
<i>B. dermatitidis</i>	16	0	0	16	8*	16	8
<i>H. capsulatum</i>	16	12	0	16	8*	16	16
<i>C. albicans</i>	0	-	-	-	-	-	-

\*These paradoxical residual titers to the heterologous or nonspecific antigens after the complete removal of the homologous antibody may represent a nonspecific complement fixation. The antigen and absorbed serum both slightly anticomplementary may remove enough complement to produce a pseudo complement fixation.

of reacting with either the two foregoing antigens or the *C. immitis* antigen whereas repeated absorption of the anti *C. immitis* serum with *C. albicans* antigen produced only a slight decrease in antibody. No absorptive qualities were shown by the antigens from *A. fumigatus* and *M. corymbifer*.

Finally, when the anti *H. capsulatum* serum was absorbed with the homologous antigen, the titer with *B. dermatitidis* complement fixation antigen was greatly reduced (Table IV). The anti *H. capsulatum* serum may then be

TABLE IV. INFLUENCE OF ABSORPTION ON COMPLEMENT FIXATION TITERS OF RABBIT ANTISERA

ANTIGEN	ANTI- <i>H. CAPSULATUM</i> SERUM						
	UNABSORBED	ABSORBED WITH					
		<i>B. DERMATITIDIS</i>	<i>H. CAPSULATUM</i>	<i>C. ALBICANS</i>	<i>C. IMMITIS</i>	<i>A. FLUORANTUS</i>	<i>M. CORYMBIFER</i>
<i>H. capsulatum</i>	64	0	0	32	32	32	64
<i>B. dermatitidis</i>	16	4	4	5	5	16	16
<i>C. albicans</i>	0	-	-	-	-	-	-
<i>C. immitis</i>	0	-	-	-	-	-	-

considered as containing a factor common to *H. capsulatum* and *B. dermatitidis* antigens. This common factor in the anti-*H. capsulatum* serum is completely responsible for the reaction with the homologous antigen, since absorption with *B. dermatitidis* produced a reduction in antibody equal to that produced by the homologous antigen. *C. albicans* and *C. immitis* antigens reduced titers only partially, whereas *A. fumigatus* and *M. corymbifer* antigens had no effect.

#### DISCUSSION

The term 'antigen' may be referred to (a) as the substance that induces the formation of antibody or (b) as the substance that reacts with antibody in a serologic test. On application of the first definition to the serologically interrelated fungi, *B. dermatitidis*, *C. albicans*, *C. immitis*, and *H. capsulatum*, the antigen from *B. dermatitidis* may be considered the most specific of the four, since it produced an antiserum that reacted only with the homologous antigen and to a much more limited extent with *H. capsulatum* antigen. However on the application of the second definition, it was the least specific serologic antigen since it fixed complement not only with its homologous antiserum but also with the three heterologous antisera. Similarly, *H. capsulatum* antigen produced a relatively specific antiserum in that it reacted about equally with its homologous antigen and the *B. dermatitidis* antigen. As a complement fixation antigen, it was conversely nonspecific since it fixed complement with the three heterologous antisera. The antigens of *C. albicans* and *C. immitis* merited the formation of antibodies that reacted with three of the four antigens studied, although as serologic antigens reactions with the homologous antisera alone were obtained.

Since absorption of a serum with a homologous antigen resulted in a reduction of titer with a heterologous antigen, and since each antigen was capable of absorbing at least some of the antibody from each of the heterologous antisera, a common factor or factors must be present in the antigens of the four species. Hence, the antigen from any of these fungi is inherently capable of reacting serologically with any one of the four antisera, although in the complement fixation tests with both pooled and individual rabbit sera the antisera from *C. albicans* and *C. immitis* did not react with heterologous antisera. However, others<sup>16, 17</sup> have reported that the sera of rabbits immunized against *B.*

*dermatitidis* fixed complement in the presence of *C albicans* antigen. No indication of a serologic cross reaction between the complement fixing antigen of *C immitis* and a heterologous antiserum has been found in the literature. Why in a previous series of experiments<sup>9</sup> cross reactions between anti *B dermatitidis* serum and *H capsulatum* yeastlike cells as antigen were not obtained is in doubt, although the reason may lie in differences in the strains used.

Of interest is the failure of *A fumigatus* and *M corymbifer* antigens to absorb significant amounts of antibodies from any of the antisera under study—a characteristic probably due to the antigenic dissimilarity between *A fumigatus* and *M corymbifer* on the one hand and *B dermatitidis*, *C albicans*, *C immitis* and *H capsulatum* on the other. This apparent lack of antigenic relationship is especially striking since *M corymbifer* is more closely allied morphologically and taxonomically to *C immitis* than either *B dermatitidis*, *C albicans* or *H capsulatum*, while *A fumigatus* is closer to the latter three species than *C immitis*.

The serologic specificity of the four related antigens was paralleled by their antigenic potency—the more specific the antigen the greater its antibody inducing powers. Thus *C albicans* and *C immitis* antigens produced the highest titered sera whereas *B dermatitidis* antigen induced sera of low titers. This high titer of a serum may partly be the basis for the reaction with heterologous antigens. Frequently the binding power of the *B dermatitidis* antigen was about the same or only slightly higher than the anticomplementary action with the result that four units of the antigen could not be used and the lot had to be discarded.<sup>14</sup>

With each of the antigens of *B dermatitidis*, *C albicans*, *C immitis* and *H capsulatum* capable of cross reaction with the sera of each other, a positive complement fixation titer in an unknown rabbit serum might theoretically be due to an antibody produced by one of the other fungi. However the antigens may still be considered to be of value in the differential diagnosis of mycotic disease, when it is emphasized (a) that fixation of complement with a heterologous antigen was generally at very low serum dilutions i.e., 1/8 and below and (b) that the titer of a serum was usually distinctly higher with the homologous than with a heterologous antigen. Several sera from an animal with a suspected mycotic disease should be collected and tested over an extended period of time, and if titers are low they should be tested with all four antigens.

#### SUMMARY AND CONCLUSIONS

Complement fixation methods were employed in the determination of the specificity of and the relationship between antigens from *Aspergillus fumigatus*, *Blastomyces dermatitidis*, *Candida albicans*, *Coccidioides immitis*, *Histoplasma capsulatum* and *Urocorymbifer*. Sera from animals experimentally infected or hyperimmunized with one of the pathogenic fungi were absorbed with each of the six fungal antigens. Also sera from experimentally infected rabbits were examined by complement fixation tests to determine the extent of their reactions with homologous and heterologous antigens.

*A. fumigatus* and *M. corymbifer* were probably antigenemically unrelated to the other four species, since they failed to absorb antibodies from any of the heterologous antisera. Although *B. dermatitidis* antigen was the least specific complement-fixing antigen of the four remaining, it produced the most specific antibody. Its antiserum reacted with the homologous antigen and slightly with the *H. capsulatum* antigen, and was absorbed by the *H. capsulatum* antigen and to a lesser extent by the *C. albicans* and *C. immitis* antigens. *H. capsulatum* antigen produced serum that reacted about equally with homologous antigen and *B. dermatitidis* antigen, and that was absorbed by *B. dermatitidis* antigen and to a lesser extent by the *C. albicans* and *C. immitis* antigens. The latter two antigens produced sera that fixed complement with and were absorbed by two of three heterologous antigens, namely, *B. dermatitidis* and *H. capsulatum* antigens.

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# THE ELECTROCARDIOGRAM OF NORMAL AND MALARIA INFECTED MONKEYS

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PRELIMINARY to the study of comparative effects of intravenous quinine dihydrochloride in normal and malaria infected monkeys,<sup>1</sup> it was incumbent upon us to establish the variations of the electrocardiogram in these two groups of animals. The available data appeared inadequate. Lloyd<sup>2</sup> noted for seventeen *Macacus rhesus* monkeys averages for the P R interval of 0.08 sec, the QRS width 0.04 sec, the Q T length 0.20 sec, and electrocardiographic patterns similar to those of man. DeWaart and Storm<sup>3</sup> published the following norms for some fifteen *Macacus irus* monkeys. P R intervals from 0.54 to 0.088 sec, QRS widths from 0.02 to 0.044 sec, and the angle of the QRS axis from +50 to +75 degrees. They established the empirical validity of the Einthoven triangle for the monkey as for man and claimed that the P and T waves were always positive and the S T intervals isoelectric as in man and not as in the dog. We considered it necessary to establish the electrocardiographic findings in a larger series of normal monkeys with the addition of the chest lead. No detailed electrocardiographic observations in malaria infected monkeys were found in the literature.

## MATERIALS AND METHODS

In this study twenty seven normal *Macaca mulatta* monkeys weighing 2 to 7 kilograms, were strapped on their backs to an animal board. Leads I, II, III, and an apical chest lead (corresponding to IV F) were obtained in the usual manner with a Cambridge electrocardiograph. Repeated tracings were taken in some instances on different days, for a total of thirty four observations, to determine the variations in single monkeys and to establish the normal electrocardiographic range. Twelve monkeys infected with *Plasmodium knowlesi*, on which normal electrocardiograms were obtained previously, had electrocardiograms with four leads similarly taken on twenty two occasions during varying stages of the infection. The course of the disease was followed by counting the number of red blood cells and the number of parasitized cells per 100 erythrocytes in the peripheral blood.

## EXPERIMENTAL RESULTS

The electrocardiograms of normal monkeys presented heart rates varying from 170 to 280 beats per minute, P R intervals from 0.06 to 0.10 sec, QRS intervals from 0.02 to 0.05 sec, Q T intervals from 0.14 to 0.20 sec, the QRS axis angle  $\alpha$  from +50 to +115 degrees. A single auricular ectopic beat was the only arrhythmia noted. We found (Table I) a flat T<sub>1</sub> in nine of thirty four observations, a negative T<sub>1</sub> in one instance, flat T in three, negative T in three, flat T<sub>2</sub> in two and negative T<sub>2</sub> in two instances. ST depression, particularly in Leads II and III of 1 mm or more was noted in eight of twenty seven monkeys. In two monkeys a negative and flat T<sub>1</sub> was found on different

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TABLE I ELECTROCARDIOGRAPHIC DEVIATIONS FROM THE AVERAGE IN NORMAL AND MALARIA-INFECTED MONKEYS

	T <sub>1</sub> FLAT	T <sub>1</sub> NEG	T <sub>2</sub> FLAT	T <sub>2</sub> NEG	T <sub>3</sub> FLAT	T <sub>3</sub> NEG	S-T DEPRESS	TOTAL CASES	TOTAL ECG
Normal	9	1	3	3	2	2	8	27	34
Malaria	6	3	1	1	0	0	3	12	22

days, in two other instances negative and flat T<sub>1</sub> waves were similarly noted, in one other instance the T<sub>1</sub> was flat and negative on two different occasions.

Malaria-infected monkeys presented heart rates in the same range as the normal monkeys with the exception of one monkey with a rate of 159 beats per minute. The atrioventricular and intraventricular conduction times had the same limits as in the normal animals. The Q-T intervals (electrical systoles) were in the upper range of normal controls, in only one severely infected monkey was a Q-T interval of 0.23 sec present, with a heart rate of 159 beats per minute. Two animals, one with moderate and one with severe parasitemia and anemia, showed marked left axis deviations,  $-40$  and  $-48$  degrees. In all others the axes were within the normal range. No arrhythmias were noted in the malaria-infected animals.

From Table I it will be seen that, in general, the proportion of negative T waves in the malaria-infected monkeys was the same as in the normal controls, nor were these negative T waves and depressed S-T segments in the malaria-infected monkeys present particularly in the more severely infected animals. In two of three animals presenting negative T<sub>1</sub> waves they were flat on other occasions, and the same variations were seen in these two monkeys before their malarial infection (Fig. 1).

#### DISCUSSION

It is clear from our observations of the electrocardiogram in twenty-seven normal *Macaca mulatta* monkeys, which is the largest group so far published, that the variability, particularly in the recovery portion (ST-T segment) of the normal tracing, is much greater than heretofore realized. Hence, if one followed the human standards of electrocardiographic criteria for the monkey, many of our findings in the malaria-infected cases would be classed as abnormal. On the other hand, comparing their tracings with the ones obtained before the infection with *P. knowlesi*, one comes to the conclusion, as did Sprague<sup>4</sup> in thousands of cases of human malaria, that the electrocardiogram is unchanged in the vast majority of instances.

Three electrocardiographic observations in malaria-infected monkeys revealed deviations from the normal variants. Two of these involved leftward changes in the axis which are difficult to interpret. The Q-T interval tended to be in the upper ranges of normal, and in one instance was somewhat prolonged, even considering the relatively slow heart rate. This might indicate the mildest form of myocardial malfunction or fatigue, which may be so expressed under other circumstances. The failure to show significant electrocardiographic abnormalities in malaria-infected monkeys is thoroughly consistent with the absence of specific pathologic lesions in the hearts of malaria-infected monkeys<sup>5</sup> and of human beings with malaria.<sup>6</sup>



In some instances of severe anemia there are seen electrocardiographic changes suggestive of coronary insufficiency and nonspecific myocardial damage presumably correlated with anoxemia fatty degeneration and even dilatation of the heart all of which may occur in severe malarial infection. The ST and T segment variations in our malaria infected monkeys however did not

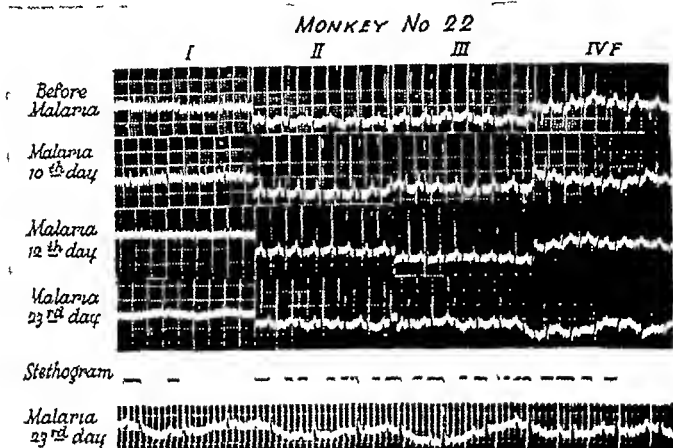


Fig 1—Before malaria the T wave was flat and ST and ST<sub>1</sub> segments were depressed in this monkey. T was either negative or biphasic on different occasions. With red blood counts of 588, 36 and 158 on the tenth, twelfth and twenty third day (day before death) of malarial infection respectively the electrocardiogram was not significantly changed except for the variations in T<sub>1</sub> as noted also before malaria and progressive prolongation of the QT interval from 0.16 to 0.18 seconds. Stethogram on the day before death was normal.

differ significantly from those of the controls. Nor did we see any prolongation of the PR interval. Our lowest attained red blood cell count 16 million per cubic millimeter may not have been low enough to cause significant anoxic electrocardiographic changes. In several instances stethographic (Fig 1) fluoroscopic and roentgenographic examinations of the heart showed no murmurs gallop rhythm or significant cardiac dilatation in severe and even fatal cases of malaria.

#### SUMMARY AND CONCLUSIONS

1 The normal electrocardiogram of the *Macaca mulatta* monkey is between that of the dog and man. The variability includes negativity of the T waves in Leads I, II and IV F and displacement of the ST segments from the iso electric line.

2 The electrocardiogram of malaria infected *Macaca mulatta* monkeys does not differ from that of the controls in the great majority of instances. The

rare exceptions include marked (left) axis deviation and slight prolongation of the Q-T interval. No typically anovemic electrocardiographic changes were demonstrated, even in monkeys showing red blood cell counts only 25 per cent of normal.

3 The absence of significant electrocardiographic alterations in monkeys infected with *P. knowlesi* is consistent with the known pathologic changes in malaria in monkeys and with the findings of others in a large series of cases of malaria in human beings.

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# LETHAL EFFECTS AND ELECTROCARDIOGRAPHIC CHANGES PRODUCED BY QUININE DIHYDROCHLORIDE IN MALARIA INFECTED MONKEYS

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INTEREST in the lethal effects of quinine on monkeys infected with malaria developed recently when it was observed that some animals died following an intravenous injection of quinine, while the same amount had no noticeable effect on other monkeys.<sup>1</sup> This observation brought to mind the controversy of fifty or sixty years ago as to whether the mortality rate was greater in cases of severe malarial fever treated with or without quinine. Deaderick<sup>2</sup> stated that the mortality rate was 25.9 per cent in 1,821 cases of blackwater fever treated with quinine while it was only 11.1 per cent in 1,006 similar cases not treated with quinine. Several workers have warned against the intravenous injection of quinine in human beings. Brahmachari<sup>3</sup> advised that in all cases of malarial fever in which the blood pressure was low, quinine dihydrochloride, when given intravenously, should be injected slowly, and accompanied by either pituitrin or adrenalin. Maxey<sup>4</sup> warned that if the patient is already suffering from circulatory embarrassment, or the severe hypotonia sometimes associated with chronic estivo autumnal infections, the added burden produced by the well intended therapeutic effort may result in a fatal outcome instead of improvement.

Large doses of quinine given intravenously to human beings directly depress the heart and lower the blood pressure.<sup>5</sup> The disturbance in heart action seems to reach its maximum in somewhat less than two minutes as 95 per cent of an intravenous dose of quinine is removed from the blood stream within five minutes.<sup>6</sup> Respiration and pulse rates are stimulated by small doses and depressed by larger ones. Death occurs usually in coma from cessation of respiration.<sup>5</sup>

In this experimental study observations were made first, to determine whether quinine dihydrochloride is fatal in smaller doses when given to monkeys with a severe malarial infection than when given to normal monkeys, and, second, to determine the electrocardiographic effects produced by quinine in these two groups of animals.

## MATERIALS AND METHODS

Thirteen normal *Macaca mulatta* monkeys, weighing from 2 to 7 kilograms, were injected intravenously with doses of quinine dihydrochloride varying from 20.6 to 82.8 mg. per kilogram. A total of nineteen injections of quinine was given to these monkeys. Eleven animals infected with malaria were given quinine dihydrochloride in doses varying from 18.7 to 41.3 mg. per kilogram. A total of twenty one injections of quinine was given to these monkeys.

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The quinine was always given intravenously over a period of thirty to forty seconds. The preparation contained 0.5 Gm of quinine dihydrochloride in each cubic centimeter, or 0.477 Gm of quinine in combination.

*Plasmodium knowlesi* was used to infect these monkeys. The course of the disease was followed by red blood cell counts and by counting the number of parasitized red cells per 500 erythrocytes in the peripheral blood. Electrocardiographic tracings were taken at varying times during the infection.

The electrocardiograms were obtained with the monkeys on their backs with each extremity tied to an animal board. Leads I, II, III, and an apical chest lead (corresponding to IV F) were made. A series of tracings was taken for the control, and during the time of the injection of the quinine lead II was recorded. Four more sets of tracings were obtained immediately, two, four, and ten minutes later. The total number of tracings made for this study, including the normal controls, was 215.

## RESULTS

Table I shows the effect of different amounts of quinine dihydrochloride when given intravenously to normal and malaria-infected animals. The maximum amount of quinine that could be given to a normal monkey without producing death was approximately 55 mg per kilogram body weight. Ten malaria-infected monkeys died with doses of quinine varying from 20.8 to 41.3

TABLE I AMOUNT OF QUININE DIHYDROCHLORIDE GIVEN INTRAVENOUSLY THAT PRODUCED DEATH IN NORMAL AND MALARIA INFECTED MONKEYS

NORMAL MONKEYS			
SURVIVED		DIED	
MONKEY	QUININE (MG/KG)	MONKEY	QUININE (MG/KG)
19	20.6	33	55.5
20	21.3	1	62.5
22	22.7	3	82.8
17	23.9		
11	25.9		
2	31.3		
32	42.5		
35	45.4		
34	45.4		
33	45.7		
31	50.0		
1	55.2		
3	55.2		

MALARIA INFECTED MONKEYS							
SURVIVED				DIED			
MONKEY	QUININE (MG/KG)	RBC (MIL LIONS)	PARASITE MIA PER 500 RBC	MONKEY	QUININE (MG/KG)	RBC (MIL LIONS)	PARASITE MIA PER 500 RBC
21	18.7	3.1	14	8	20.8	3.04	265
13*	20.6	3.46	69	21	22.7	2.04	36
19	20.6	6.48	62	22	22.7	1.66	24
22	22.7	3.6	12	7*	27.5	1.9	237
22	22.7	1.58	37	13	27.5	1.9	161
5	26.9	-	62	28*	27.5	1.21	122
28*	27.0	3.39	3	10	31.4	3.24	154
7	27.5	4.1	127	30*	35.5	-	9
30*	28.4	-	16	11	37.5	-	121
12	37.5	-	0	16	41.3	-	51
16*	41.3	-	14				

\*No electrocardiographic record

mg per kilogram body weight Six of these ten animals died following the injection of 27.5 mg or less of quinine per kilogram of body weight The malaria infected monkeys that succumbed to the quinine had severe anemia and usually a high degree of parasitemia Ten monkeys with less severe anemia and fewer parasites in the circulating blood survived a dose of quinine of equal size (Table I)

TABLE II. LETHAL EFFECT OF QUININE DIHYDROCHLORIDE ON MONKEYS INFECTED WITH P. KNOWLESII

MONKEY	WEIGHT (KG)	EXPERIMENTAL DAY	RBC (MILLIONS)	PARASITE MIA PER 100 RBC	QUININE (MG)	RESULTS
21	4.0	10	5.8	10	75	Survived
		12	4.2	14	75	Survived
		13	3.1	14	75	Survived
		19	2.04	36	75	Died immediately
22	3.3	10	7.99	89	75	Survived
		12	3.6	12	75	Survived
		22	1.62	35	75	Survived
		23	1.56	37	75	Survived
		24	1.66	24	75	Died 30 min later
375	-	9	3.58	129	75	Survived
		21	1.29	45	75	Died immediately
7	3.63	10	4.1	127	100	Survived
		12	1.90	237	100	Died immediately
28	-	2	-	3	100	Survived
		3	-	10	100	Survived
		4	-	46	100	Survived
		6	3.39	3	100	Survived
		11	1.21	12	87	Died immediately
16	3.63	6	-	14	150	Survived
		7	-	1	150	Survived
		12	-	51	150	Died immediately

Six monkeys with malaria were given the same amount of quinine intravenously one to four times preceding the last injection of the identical amount of quinine which resulted in their immediate deaths (Table II). These monkeys lost some weight during their disease, however, this factor alone apparently cannot account for their response to the same quantity of quinine at different intervals. Monkey 28 survived 100 mg (27.4 mg per kilogram) of quinine on four different occasions and died following the fifth injection of 87 mg (27.1 mg per kilogram). The anemia was severe at the time of death in each of these six monkeys. Fig. 1 shows the correlation between the anemia, parasitemia, and the periodic intravenous injections of quinine in Monkey 7.

The monkeys given the larger amounts of quinine first showed respiratory difficulties beginning about sixty seconds following the time of the injection. Respirations markedly decreased and frequently stopped for varying intervals following which time the monkeys gasped irregularly. During this period cyanosis and slowing of the heart occurred and in such animals death usually followed in two to ten minutes. With a slightly smaller dose of quinine the monkeys developed severe convulsions preceded and followed by twitchings of different groups of muscles. Cyanosis was frequently marked during the apnea and convulsions. Some of the monkeys that had severe convulsions died

five to fifteen minutes later. The animals that survived the convulsions were unable to stand when returned to their cages thirty to sixty minutes following the injection of the quinine. Gradually these monkeys improved and twelve to eighteen hours later appeared normal. With the smaller doses of quinine no clinical changes were observed.

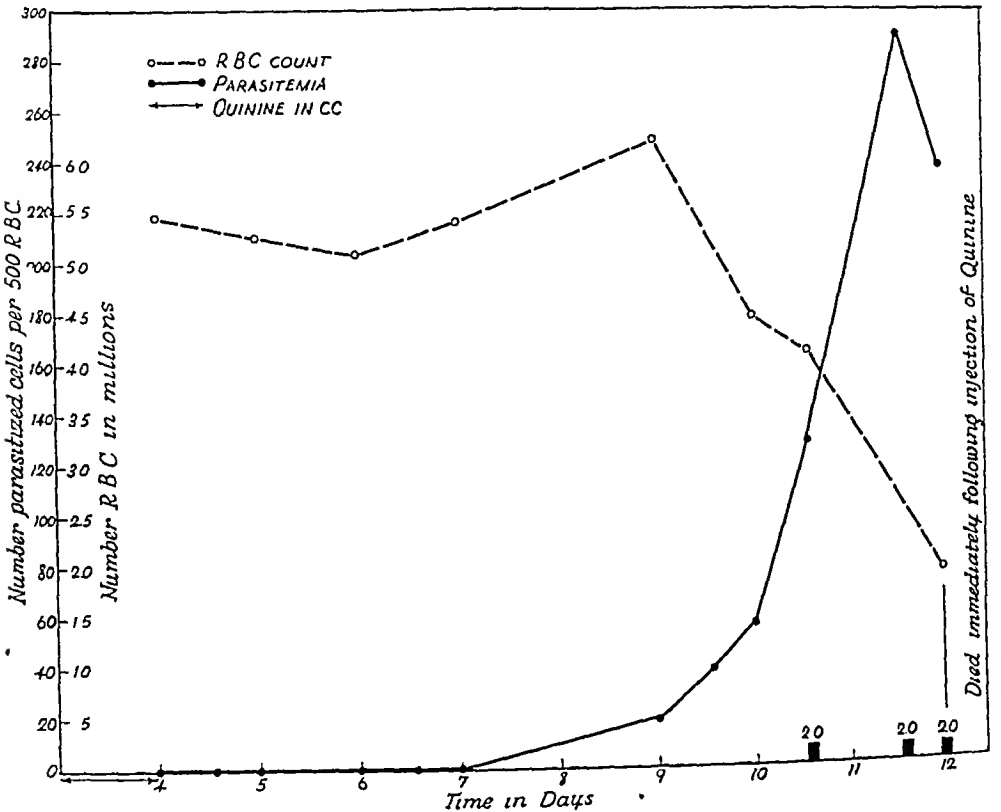


Fig 1—Monkey 7. There is a progressive and rapid increase in the degree of parasitemia and a corresponding decrease in the number of red cells in the typical case of malarial infection in the monkey. Death occurred immediately following the third intravenous injection of 2 cc of quinine. Death following the injection of quinine may be correlated with the anemia.

The electrocardiographic effects of quinine given intravenously in both normal and malaria-infected monkeys were usually maximum immediately after the completion of the injection. All the intervals usually returned to normal in six to thirty minutes, with the exception of the Q-T interval. This was prolonged for indefinite periods of time following larger doses of quinine in both normal and malaria-infected monkeys. Not infrequently, however, the Q-T interval was 0.1 to 0.2 seconds shorter than the control duration at ten minutes after the injection of small doses of quinine in normal monkeys (Fig 2). ST segment deviations and T-wave reversals occurred after quinine in both normal and malaria-infected animals.

The effects of near-equivalent doses of quinine in three representative normal and subsequently malaria-infected monkeys are shown in Table III. In

are compared in Fig. 2 and the maximum deflections of the aortic flow and intracardiac output are shown in Table III of the electrical system and the deflection in the heart rate. In general it may be said that the aortic flow

MONKEY No. 21  
Quinine dithiocarbamate 33 mg/kg

I II III IV/F

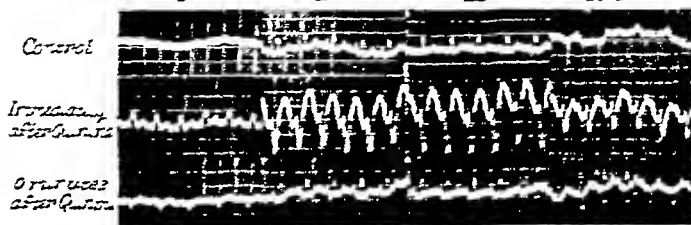


Fig. 2. A series of four ECG-like tracings showing the effect of quinine dithiocarbamate on the heart rate and rhythm. The top tracing is labeled 'Control' and shows a regular, low-amplitude waveform. The second tracing is labeled 'Irregularly irregular' and shows a highly irregular, high-amplitude waveform. The third tracing is labeled 'Organic' and shows a regular, high-amplitude waveform. The bottom tracing is labeled 'Organic' and shows a regular, high-amplitude waveform.

Quinine dithiocarbamate was administered to the monkey at a dose of 33 mg/kg. The results are shown in Table III. The heart rate and rhythm were recorded before and after administration. The heart rate was 72 beats/min before and 74 beats/min after. The rhythm was regular before and irregular after. The aortic flow and intracardiac output were also recorded. The aortic flow was 1.5 l/min before and 1.6 l/min after. The intracardiac output was 1.5 l/min before and 1.6 l/min after.

TABLE III. THE EFFECT OF QUININE DITHIOCARBATE ON THE HEART RATE AND RHYTHM OF MONKEYS.

No.	Sex	Age	Weight (kg)	Dose (mg/kg)	Time (hr)	Heart Rate (beats/min)			Rhythm
						Pre	Post	Post	
1	M	1	1.5	33	0	72	74	74	Reg.
2	M	1	1.5	33	0	72	74	74	Reg.
3	M	1	1.5	33	0	72	74	74	Reg.
4	M	1	1.5	33	0	72	74	74	Reg.
5	M	1	1.5	33	0	72	74	74	Reg.
6	M	1	1.5	33	0	72	74	74	Reg.
7	M	1	1.5	33	0	72	74	74	Reg.
8	M	1	1.5	33	0	72	74	74	Reg.
9	M	1	1.5	33	0	72	74	74	Reg.
10	M	1	1.5	33	0	72	74	74	Reg.
11	M	1	1.5	33	0	72	74	74	Reg.
12	M	1	1.5	33	0	72	74	74	Reg.
13	M	1	1.5	33	0	72	74	74	Reg.
14	M	1	1.5	33	0	72	74	74	Reg.
15	M	1	1.5	33	0	72	74	74	Reg.
16	M	1	1.5	33	0	72	74	74	Reg.
17	M	1	1.5	33	0	72	74	74	Reg.
18	M	1	1.5	33	0	72	74	74	Reg.
19	M	1	1.5	33	0	72	74	74	Reg.
20	M	1	1.5	33	0	72	74	74	Reg.
21	M	1	1.5	33	0	72	74	74	Reg.
22	M	1	1.5	33	0	72	74	74	Reg.
23	M	1	1.5	33	0	72	74	74	Reg.
24	M	1	1.5	33	0	72	74	74	Reg.
25	M	1	1.5	33	0	72	74	74	Reg.
26	M	1	1.5	33	0	72	74	74	Reg.
27	M	1	1.5	33	0	72	74	74	Reg.
28	M	1	1.5	33	0	72	74	74	Reg.
29	M	1	1.5	33	0	72	74	74	Reg.
30	M	1	1.5	33	0	72	74	74	Reg.
31	M	1	1.5	33	0	72	74	74	Reg.
32	M	1	1.5	33	0	72	74	74	Reg.
33	M	1	1.5	33	0	72	74	74	Reg.
34	M	1	1.5	33	0	72	74	74	Reg.
35	M	1	1.5	33	0	72	74	74	Reg.
36	M	1	1.5	33	0	72	74	74	Reg.
37	M	1	1.5	33	0	72	74	74	Reg.
38	M	1	1.5	33	0	72	74	74	Reg.
39	M	1	1.5	33	0	72	74	74	Reg.
40	M	1	1.5	33	0	72	74	74	Reg.
41	M	1	1.5	33	0	72	74	74	Reg.
42	M	1	1.5	33	0	72	74	74	Reg.
43	M	1	1.5	33	0	72	74	74	Reg.
44	M	1	1.5	33	0	72	74	74	Reg.
45	M	1	1.5	33	0	72	74	74	Reg.
46	M	1	1.5	33	0	72	74	74	Reg.
47	M	1	1.5	33	0	72	74	74	Reg.
48	M	1	1.5	33	0	72	74	74	Reg.
49	M	1	1.5	33	0	72	74	74	Reg.
50	M	1	1.5	33	0	72	74	74	Reg.
51	M	1	1.5	33	0	72	74	74	Reg.
52	M	1	1.5	33	0	72	74	74	Reg.
53	M	1	1.5	33	0	72	74	74	Reg.
54	M	1	1.5	33	0	72	74	74	Reg.
55	M	1	1.5	33	0	72	74	74	Reg.
56	M	1	1.5	33	0	72	74	74	Reg.
57	M	1	1.5	33	0	72	74	74	Reg.
58	M	1	1.5	33	0	72	74	74	Reg.
59	M	1	1.5	33	0	72	74	74	Reg.
60	M	1	1.5	33	0	72	74	74	Reg.
61	M	1	1.5	33	0	72	74	74	Reg.
62	M	1	1.5	33	0	72	74	74	Reg.
63	M	1	1.5	33	0	72	74	74	Reg.
64	M	1	1.5	33	0	72	74	74	Reg.
65	M	1	1.5	33	0	72	74	74	Reg.
66	M	1	1.5	33	0	72	74	74	Reg.
67	M	1	1.5	33	0	72	74	74	Reg.
68	M	1	1.5	33	0	72	74	74	Reg.
69	M	1	1.5	33	0	72	74	74	Reg.
70	M	1	1.5	33	0	72	74	74	Reg.
71	M	1	1.5	33	0	72	74	74	Reg.
72	M	1	1.5	33	0	72	74	74	Reg.
73	M	1	1.5	33	0	72	74	74	Reg.
74	M	1	1.5	33	0	72	74	74	Reg.
75	M	1	1.5	33	0	72	74	74	Reg.
76	M	1	1.5	33	0	72	74	74	Reg.
77	M	1	1.5	33	0	72	74	74	Reg.
78	M	1	1.5	33	0	72	74	74	Reg.
79	M	1	1.5	33	0	72	74	74	Reg.
80	M	1	1.5	33	0	72	74	74	Reg.
81	M	1	1.5	33	0	72	74	74	Reg.
82	M	1	1.5	33	0	72	74	74	Reg.
83	M	1	1.5	33	0	72	74	74	Reg.
84	M	1	1.5	33	0	72	74	74	Reg.
85	M	1	1.5	33	0	72	74	74	Reg.
86	M	1	1.5	33	0	72	74	74	Reg.
87	M	1	1.5	33	0	72	74	74	Reg.
88	M	1	1.5	33	0	72	74	74	Reg.
89	M	1	1.5	33	0	72	74	74	Reg.
90	M	1	1.5	33	0	72	74	74	Reg.
91	M	1	1.5	33	0	72	74	74	Reg.
92	M	1	1.5	33	0	72	74	74	Reg.
93	M	1	1.5	33	0	72	74	74	Reg.
94	M	1	1.5	33	0	72	74	74	Reg.
95	M	1	1.5	33	0	72	74	74	Reg.
96	M	1	1.5	33	0	72	74	74	Reg.
97	M	1	1.5	33	0	72	74	74	Reg.
98	M	1	1.5	33	0	72	74	74	Reg.
99	M	1	1.5	33	0	72	74	74	Reg.
100	M	1	1.5	33	0	72	74	74	Reg.

The effects on the three electrocardiographic intervals of the various doses of quinine used in both the normal and malaria-infected animals respectively are plotted in Fig 3. By contrasting the two groups, a graphic illustration is provided to show that smaller doses of quinine produce greater cardiodepressive effects in malaria-infected monkeys than in normal animals. These electro-

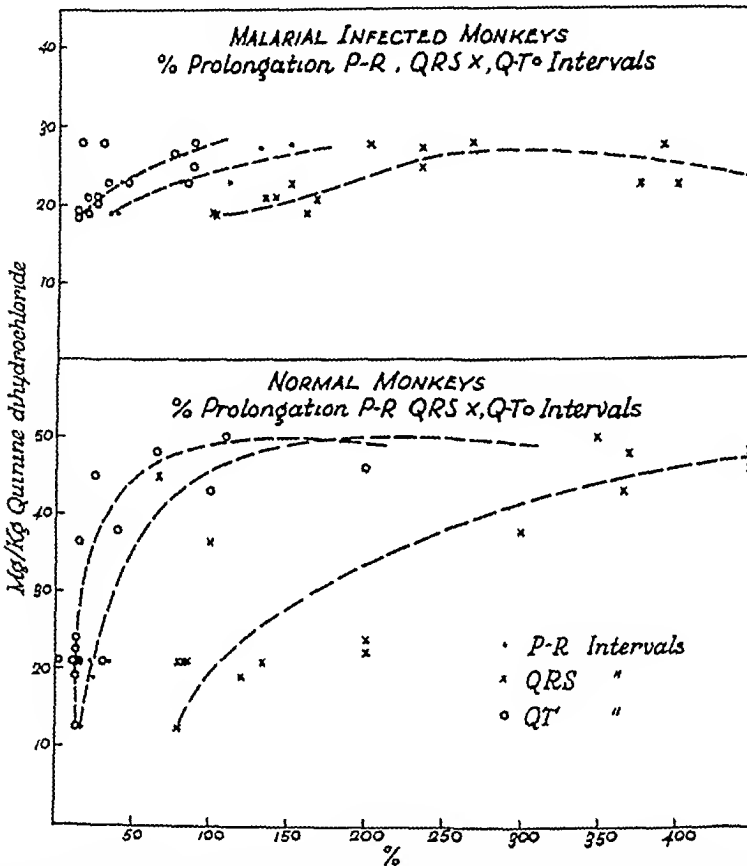


Fig 3—The variation of the prolongation by varying doses of quinine of the atrioventricular and ventricular conduction times and the Q-T interval in normal and malarial monkeys. Much larger doses of quinine usually failed to prolong the electrocardiographic intervals in normal monkeys than smaller doses did in malaria-infected animals.

cardiographic findings thus corroborate the clinically observed effects on the respiratory and neuromuscular systems and the higher mortality in malaria-infected monkeys than in the normal monkeys following equivalent doses of quinine.

It should be emphasized that the electrocardiographic changes induced by quinine in normal and infected monkeys vary only in degree and not in kind. The pattern following large and sublethal doses is as follows: prolongation of the intervals, slowing of the rate, auricular standstill with increasingly slower idioventricular rhythm, not infrequently periods of ventricular tachycardia and ventricular ectopic beats, rarely auricular fibrillation or flutter with varying A-V block. All of these changes are reversible in animals that recover.



from these severe cardiac effects as in those that survive respiratory arrest and convulsions lasting several minutes

The electrocardiographic findings in monkeys that died were typified by increasing intraventricular block and ventricular slowing and asystole (Fig 4). Frequently, however, respiratory failure was followed by reversal of the severe

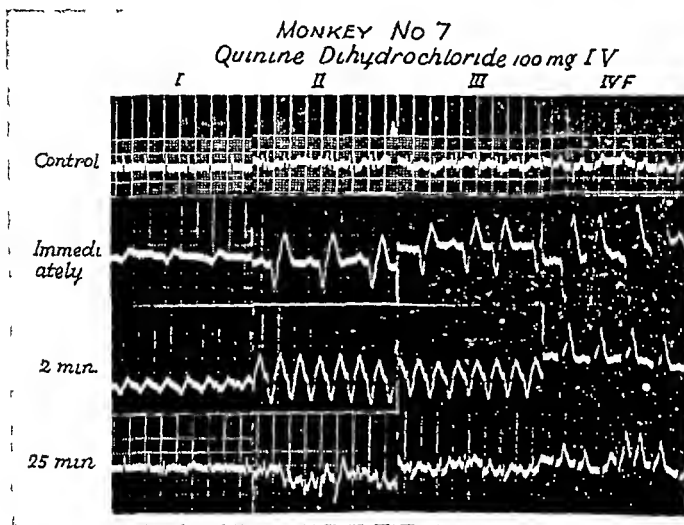


Fig 4—Monkey 7. Partially reversible electrocardiographic effects of a relatively small dose (27.7 mg per kilogram) of quinine in a malaria infected monkey (parasitemia 1.7 per 500 R-B-C, red blood count, 4.1 million). Marked slowing of the rate, ventricular ectopics preceding and following ventricular tachycardia, reversal of the conduction defects twenty five minutes after quinine administration.

electrocardiographic manifestations, so that only slow sinus rhythm with fairly short P R QRS, and Q T intervals accompanied or followed by five to twenty minutes 'clinical death'.

The rapid accession and recession of cardiac effects produced by quinine also noted by previous observers\* were emphasized in our experiments. As an example, nearly identical conduction defects followed small (e.g. 20 mg per kilogram) doses of quinine repeated in ten minutes at which time the effects of the first dose were usually completely gone. Larger (e.g. sublethal) doses required up to thirty or more minutes for complete reversal. Arrhythmic arrhythmias and prolonged Q T intervals tended to persist following such dosage for the longest period of time. Gallop rhythm was noted stethographically only once in seven observations. Fluoroscopic and roentgenologic studies in a few monkeys revealed no significant enlargement of the cardiac chambers.

## DISCUSSION

In this study no attempt was made to establish the minimal lethal dose of quinine, either for normal monkeys or for animals in varying stages of malarial infection. Although the number of animals used in these experiments is too small for statistical purposes, the data would indicate that a monkey with a severe malarial infection is more susceptible to the action of quinine than a monkey with a less severe malarial infection. It would appear from our data that the susceptibility to quinine is related more closely to the anemia than it is to the degree of parasitemia. Of course, the anemia in malaria results from the destruction of the red blood cells by the parasites. In an untreated animal there is a close correlation between the degree of parasitemia and the anemia. However, when quinine is given the parasitemia may decrease without an immediate increase in the number of red blood cells.

It is well known that the salts of quinine frequently are lethal when given intravenously. McCarrison and Cornwall in 1918,<sup>7</sup> while studying the pharmacodynamics of quinine in sheep, showed that "all the salts of quinine employed caused a profound fall of blood pressure not accompanied by a cessation, or even much diminution in the strength, of the heart's beat, except in the two instances in which the respiratory center failed." The fall of blood pressure is usually recovered from in four or five minutes but the period of cardiovascular depression may last for a considerable time.<sup>8</sup> These investigators also emphasized the fact that "the respiratory center is more gravely affected than the cardiac center." Moxey<sup>9</sup> noted in human patients given rapid intravenous injections of quinine symptoms simulating the early findings in our monkeys: Dizziness, sweating, hypotension, dilated pupils, twitchings of the face lasting three to ten minutes, and possible convulsions, were, in his experience, danger signals.

The electrocardiographic findings in normal and malaria-infected monkeys have been reported by us in a previous paper.<sup>8</sup> There is little variation from normal in the electrocardiographic pattern of monkeys with a severe malarial infection. The changes in a monkey with a severe malarial infection given a lethal dose of quinine are similar to those in a normal monkey given a lethal dose of quinine, although the quantity of quinine given to the malaria-infected animal is only about one-half that necessary to produce death in the normal monkey. The conduction times and electrical systole are more markedly prolonged in quinine-treated monkeys with a severe malarial infection than in normal animals and, also, those monkeys with a less severe malarial infection. The occasional shortening of the Q-T interval seen ten minutes after the injection of quinine parallels the same finding in isolated rabbit hearts following quinidine.<sup>9</sup>

The questions may be asked: Is the effect of quinine only directly on the myocardium and the conduction system, or are the resulting electrocardiographic changes aggravated by anoxemia and the depressive action of quinine on the medullary centers? It would appear to us that anoxemia is not the primary mechanism in the cardiac response since monkeys with a severe malarial infection are anoxic, and then electrocardiographic responses are essentially

the same as those of normal monkeys. The direct cardiac effects of quinine and the relative coronary insufficiency resulting from anemia, hypotension, and respiratory insufficiency, however, may be additive.

A second problem in the mechanism of death in these malaria infected monkeys is the relationship of anemia to the action of quinine on the respiratory center. The manner of death is the same in monkeys with a severe malarial infection given a small amount of quinine as it is in a normal monkey given twice as much quinine. In man it is well known that the respiratory volume and the cardiac output are increased in cases of severe anemia. This implies increased work and fatigue of both the respiratory center and the heart. Large doses of quinine markedly depress both the heart and respiratory centers, especially in monkeys with a severe anemia. The electrocardiographic changes in dogs with a severe and irreversible anoxia<sup>10</sup> are similar to our findings in monkeys following a lethal dose of quinine. As previously stated, the electrocardiograms were essentially normal in our monkeys with a severe malarial infection, although they were anoxic. This apparent discrepancy in the electrocardiographic changes in these dogs and monkeys may be explained by the fact that the degree of anemia in our malaria infected monkeys apparently had not reached the irreversible stage, while the dogs studied by Swann<sup>10</sup> were in extremis at the time the tracings were obtained. Experiments are now in progress to study the relation of anemia to the effects produced by quinine.

#### SUMMARY

1 Monkeys with a severe anemia resulting from *P. knowlesi* infection succumb to a smaller dose of quinine given intravenously than normal animals or those with a less severe anemia.

2 The electrocardiographic changes following equivalent doses of quinine in malaria infected monkeys follow the same pattern as in normal monkeys, but show evidence of greater cardiodepression.

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## A NEW INTRAMUSCULAR PREPARATION OF QUINIDINE (QUINIDINE GLUCONATE)\*

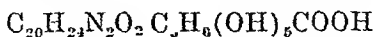
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THE object of this communication is to report our experience with the use of an intramuscular preparation of quinidine which we believe has certain advantages over that at present available. The value of quinidine in the treatment of certain cardiac arrhythmias is well recognized. In most of these, the preparation available for oral use, quinidine sulphate, is adequate. However, in certain acute or paroxysmal arrhythmias, the abnormal rate and rhythm may cause cardiac pain, intense palpitation, dyspnea, vomiting, a shocklike state, and even death. In these conditions where quinidine is of recognized efficacy, parenteral administration, because of its more rapid absorption and certainty of effect, has certain advantages over oral administration. In addition, other indications for parenteral administration are vomiting and diarrhea, moderate or severe grades of congestive failure when absorption by mouth is slow and uncertain, and the presence of any condition (postoperative, for example) which might prevent the patient from taking food by mouth.

The intravenous preparations of quinidine which are available are quinidine hydrochloride and quinidine lactate. Our experience with the intravenous preparations is limited to relatively few patients because of their possible harmful effect.

We have had a rather extensive experience with the intramuscular preparations of quinidine over a period of years. We have found the intramuscular method to be a satisfactory and a relatively safe one for administration of this drug. Sagall, Horn, and Riseman<sup>1</sup> have shown that following an intramuscular injection of 5 grams of quinidine hydrochloride, a definite response is observed in fifteen minutes and occasionally in five minutes. The maximum effect of the quinidine injected intramuscularly was obtained in one and one-half hours. As far as we are aware, the only intramuscular preparation at present available is that reported by Stumek, Riseman, and Sagall,<sup>2</sup> in which quinidine hydrochloride is buffered with urea and antipyrine. We have found this preparation to be quite satisfactory. However, in some of the lots which we have had prepared, the salt has settled out and some of the injections have been painful.

We have recently used quinidine gluconate for intramuscular injection and have found the results obtained to be quite satisfactory. The formula for this preparation is



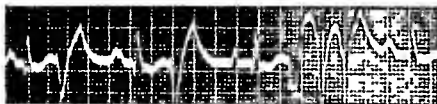
From The Division of Cardiology, Philadelphia General Hospital and The Robinette Foundation, University of Pennsylvania.

This work was aided by a grant from the United States Public Health Service.

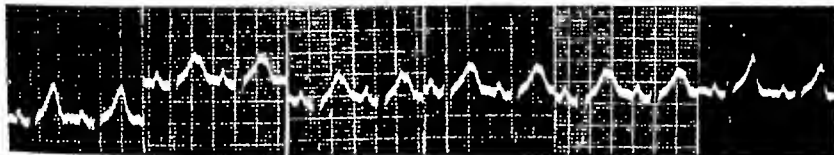
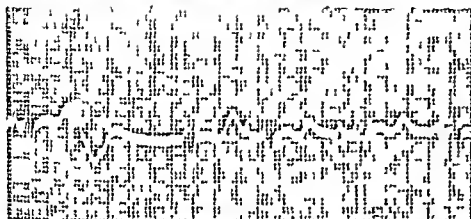
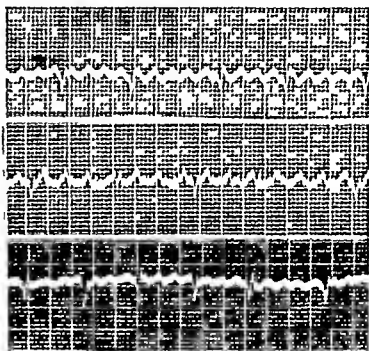
Received for publication May 24, 1949.

\*The drug was supplied by the Physician's Drug and Supply Company, Philadelphia, Pa.

A



B



C

Fig 1—1 G. W. 62 year old man. Diagnosis: arteriosclerotic heart disease congestive heart failure and diabetes. (Lead CR<sub>2</sub>) Upper strip shows evidence of digitalis intoxication. Many extrasystoles are noted three of them occurring in succession. Lower strip four hours after 7½ grains of quinidine gluconate have been given intramuscularly shows only occasional ventricular extrasystoles and a return to a predominantly normal rhythm.

B. M. D. 48 year old woman. Diagnosis: rheumatic heart disease mitral stenosis cardiac enlargement and auricular fibrillation. (Lead CR<sub>2</sub>) Upper control strip shows auricular fibrillation with a ventricular rate of about four hundred per minute. Middle strip one hour after 6 grains of quinidine gluconate had been given intramuscularly shows that the auricular rate has slowed to three hundred per minute with the production of an auricular flutter. The slowing of the auricular rate in auricular fibrillation is a characteristic quinidine effect. Lower strip eleven hours after quinidine gluconate had been given shows a return of the auricular fibrillation.

C. A. D. 48 year old man. Heart normal. Lead CR<sub>2</sub> was taken. Seven and one half grains of quinidine gluconate were administered intramuscularly and electrocardiograms repeated at fifteen minute intervals for three hours and at one hour intervals thereafter. The segments of the record are from right to left: control fifteen minutes thirty minutes two hours two and three quarter hours and twenty four hours after quinidine was administered. Note the initial effect of quinidine action observed in the tracing taken fifteen minutes after injection. Note the notched T wave and the slight lengthening of the QT interval which continues for several hours. The tracing taken twenty four hours after the injection shows a return to normal with absence of quinidine effects.

The molecular weight is 520.55, it contains anhydrous quinidine 62.3 per cent and gluconic acid 37.7 per cent. It occurs as a white dextrorotatory powder which is soluble in nine parts of water. The solution is neutral or slightly alkaline in reaction.<sup>3</sup>

This preparation has been used by us in various cardiac states in fifteen patients some of whom received as many as ten injections of 150 mg. at varying intervals. This preparation appears to have some advantages over that mentioned. The solution is stable, the preparation is relatively simple in type, it results in no irritation at the site of the injection, and the administration of

other compounds (for example, antipyrin, the action of which may be unwanted and undesirable) is not necessary. Characteristic quinidine effects were obtained clinically. These consisted of abolition of ventricular extrasystoles, slowing of the auricular rate in auricular fibrillation, and the production of characteristic changes in the T waves and Q-T intervals in patients with normal sinus rhythm. We have obtained observable effects within fifteen minutes following the injection of a 5 to 7½ grain dose of the drug (Fig 1, A, B, and C).

#### SUMMARY

Our experience with an intramuscular preparation of quinidine (quinidine gluconate) has been reported. The solution is stable, is simple in type, and results in no irritation at the site of the injection. A characteristic quinidine effect is observed in a relatively short period of time after injection, as early as fifteen minutes after a dose of 5 to 7½ grains. Absorption by the intramuscular route is certain, uniform, and relatively safe. This route of administration is suggested for those patients in whom a rapid effect is required or in whom its administration by the oral route is not considered feasible.

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# STUDY OF COMPLETE PARENTERAL ALIMENTATION ON DOGS

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THE present study was undertaken in an effort to improve the nutritional status of patients who cannot receive food by mouth but who must be fed parenterally. Such patients require a nutrient supply not only adequate in caloric content but also one which contains sufficient water, minerals, vitamins, carbohydrate, protein and fat. Each of these six nutritional elements requires the presence of the other five before it can be properly utilized.

Intravenous feeding of glucose saline, some of the vitamins and amino acids or protein hydrolysate has been common practice for some time but has not proved to be completely satisfactory because of the lack of fat. It has been difficult to obtain a fat emulsion suitable for intravenous use and the literature contains only five reports<sup>1-5</sup> of attempts to use parenteral alimentation using all three of the energy producing foodstuffs. In these reports either the diet was incomplete or the period of feeding too short for adequate evaluation of results.

Recent work<sup>6</sup> has led to the development of a stable fine fat emulsion which is believed to be suitable for intravenous use and should overcome the difficulties described. A complete intravenous diet was prepared using this emulsion and a protein hydrolysate. It was administered to dogs over a period of ten weeks during which time they received food exclusively by vein.<sup>7</sup> Results were generally very satisfactory but the animals showed intermittent hematuria and a mild anemia.

It was felt that the hematuria might have been due to the mechanical difficulties in voiding. The present study was carried out in an attempt to avoid the previous difficulties and demonstrate the possibility of maintaining animals completely healthy by complete parenteral alimentation.

## EXPERIMENTAL PROCEDURES

The fat emulsion used in this experiment was prepared as described in the previous communication.<sup>6</sup> However its composition was altered slightly. It is shown in Table I.

Five healthy adult dogs were used in this experiment. The animals were confined in individual metabolism cages which permitted a quantitative collection of urine. In the control period they were fed a complete basal diet (Table II) which furnished 80 calories per kilogram of body weight per day of which 50 per cent came from carbohydrate, 16 per cent from protein, and 34 per cent from fat. Immediately following this period, four animals (Dogs B, R, C, and M) were given a diet containing the same amount of carbohydrate, protein, and fat, but it was infused exclusively by vein. Carbohydrate was furnished in the form of glucose, protein in the form of Amigen<sup>8</sup> and fat as 10 per cent olive oil emulsion.

From the Department of Physiology Vanderbilt University Medical School

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Amigen a protein hydrolysate was furnished through the courtesy of Dr. Warren M. Cox, Jr. Mead Johnson & Company Evansville Ind.

TABLE I COMPOSITION OF FAT EMULSION

CONSTITUENT	AMOUNT
Pure olive oil*	10 0 Gm %
Span 20	0 5 Gm %
Asolectin	0 4 Gm %
Sodium cholate	0 1 Gm %
Glucose	5 0 Gm %
Vitamin A†	770 units
Vitamin D†	110 units
Alpha tocopherol acetate	3 5 mg
Distilled water	85 ml

\*Pure virgin olive oil

†Supplied as Oleum Percomorphum a product of Mead Johnson &amp; Company Evansville

Ind

TABLE II COMPLETE BASAL DIET

CONTENT	AMOUNT/KG PER DAY	CAL /KG PER DAY	% CALORIES
Sucrose	10 00 Gm	40 0	50 0
Casein	3 30 Gm	13 2	16 0
Yeast	0 90 Gm	—	—
Cellulose	0 90 Gm	—	—
Salt mixture*	0 42 Gm	—	—
Lard	3 00 Gm	27 0	34 0
Vitamin A	250 U S P units	—	—
Vitamin D	36 U S P units	—	—
Total	18 52 Gm	80 2	

\*Wesson's salt mixture\*

Minerals and vitamins other than A, D, and E were supplied also. The composition and amount of mineral and vitamin mixtures infused into the animals in this study are shown in Tables III and IV. Water was allowed by mouth ad libitum and liver extract\* and folic acid† were given intramuscularly every week. The fifth dog (Dog Y) received the same amount of carbohydrate, protein, minerals, and vitamins but without the fat. The caloric intake of Dog Y during the parenteral feeding period was inadequate (53 calories per kilo gram per day).

TABLE III SALT MIXTURE

CONSTITUENT	GM /KG PER DAY
Sodium chloride	0 400
Potassium iodide	0 001
Calcium gluconate	0 250
Magnesium chloride	0 025
Potassium chloride	0 02
Ferric chloride	0 003
Copper chloride	0 0007
Potassium acid phosphate	0 048
Sodium phosphate, dibasic	0 150

TABLE IV VITAMIN MIXTURE

CONSTITUENT	MG /KG PER DAY
Thiamine HCl	0 20
Riboflavin	0 10
Pyridoxine HCl	0 06
Calcium pantothenate	0 20
Choline chloride	10 00
Inositol	2 00
Nicotinamide	2 00
Hykinone	0 10

\*Liver Injection U S P obtained from Division of Wilson and Company Inc Chicago  
 III One milliliter (10 U S P units) was given weekly

†Folvite a product of Lederle Laboratories Division American Cyanamid Company New York, N Y Two milliliters (15 mg per milliliter) were given every week



The effects of complete parenteral alimentation on hematologic changes, body weight, nitrogen balance, "water balance", liver function, total plasma protein, plasma nonprotein nitrogen, and urinary sugar and albumin were followed. The same studies also were carried out for three or four weeks prior to the injections.

Methods of chemical determinations and hematologic study performed in this experiment are as follows. The nitrogen content of urine and diet was determined by the semi-micro Kjeldahl method. The nitrogen content of feces was calculated as 12 per cent of the urinary nitrogen excretion based on the previous experimental results.<sup>2</sup> The nitrogen balance in the three day period was determined. The "water balance" was simply measured by the differences between the volume of fluid intake (water by mouth or water by mouth plus infused fluid) and the volume of urinary output. It also was conducted over a three day period. A blood sample for hematologic study was drawn on Monday morning before the infusion started in all instances (approximately thirty to forty hours after the previous injection of the six day a week schedule). Blood hemoglobin was determined by Sahli's method. Red and white blood cells were counted in the accepted manner and the hematocrit was obtained by centrifuging the oxalated blood in a Winthro tube for half an hour at 2500 revolutions per minute. Body weight was obtained just prior to the blood sampling for hematologic study every Monday.

The methods used for determining total plasma protein, plasma nonprotein nitrogen, rose bengal dye clearance test, serum phosphatase and urinary sugar and albumin were the same as described in the previous communication.<sup>4</sup>

A solution containing 10 per cent Amigen, 15 per cent glucose and the required minerals and vitamins was mixed and infused as one solution. The olive oil emulsion was infused separately and in all instances was infused before the other mixture. Daily infusion was completed in approximately nine to ten hours (three to four hours for fat emulsion and six to seven hours for Amigen, glucose, vitamins and minerals). The infusion rate of glucose, protein and fat was approximately 1.4, 0.55 and 0.86 Gm per kilogram of body weight per hour respectively.

During infusion, the animals remained in the individual cages. The use of the swivel of Jacobs<sup>10</sup> with modification permitted the animal complete freedom of movement during the infusion. Infusions were carried out by introducing polyethylene tubing\* (0.025 by 0.004 in.) into the external jugular vein. The tubing was left in the vein for two to five weeks. The dogs were infused for four weeks and then sacrificed for histologic study.

## RESULTS

*General Appearance*—All animals (Dogs P, R, C, and M) which received complete parenteral alimentation including the fat remained healthy, lively and in good spirits throughout. Their hair was smooth and shining. However, Dog Y, which received no fat, appeared very emaciated and apathetic. Its hair was very coarse and dry and epilation was starting to occur on the hind legs. The conditions are shown in Figs. 1 and 2.

*Hematologic Studies*—In Table V are shown the hematologic values of the five animals (Dogs B, R, C, M, and Y) determined during the control period. Table VI presents the average deviation of the weekly hematologic findings of Dogs B, R, C, and M during the experimental period from those determined during the period of control. The deviations of the weekly findings during the experimental period of Dog Y are also shown in Table VI.

\*Polyethylene tubing was obtained from the Irvington Varnish and Insulator Company, Irvington, N. J.

The differences in white cell count and hemoglobin determination in Dogs B, R, C, and M during the entire four-week period of complete parenteral alimentation with the fat were not significant. However, these dogs showed a slight fall in red cell count after the fourth week and a slight fall in hemato



Fig 1—A photograph of Dog B which was taken at the end of the four-week period of complete parenteral alimentation including the fat

TABLE V \* HEMATOLOGIC VALUES DURING THE CONTROL PERIOD†

DOG	BLOOD PICTURE			
	RBC (MILLION PER CU MM)	WBC (PER CU MM)	HB (GM %)	HEMATOCRIT (%)
B	6.03	17,350	14.2	44.5
R	6.96	14,517	13.6	44.5
C	6.04	15,400	13.9	38.0
M	5.27	9,988	13.7	37.0
Y	4.58	10,180	12.3	34.0

\*Acknowledgment is made to Dr Margaret P. Martin, Vanderbilt University Medical School, for statistical analysis of the data presented in Tables V, VI, and VII.

†For red blood cell count and white blood cell count the average of three or four weekly observations for each dog was computed. For hemoglobin and hematocrit the last value of the three or four weekly observations for each dog was used.

crit after the third week of infusion which is statistically significant. Dog Y, the animal which received parenteral alimentation without fat, also showed a slight reduction in hematocrit after the third week and a marked reduction after the fourth week of infusion.



Fig. —A photograph of Dog Y which was taken at the end of the four week period of parenteral alimentation without the fat.

TABLE VI DEVIATION FROM THE VALUES DETERMINED DURING THE CONTROL PERIOD

WEEK (EXPERIMENTAL)	BLOOD PICTURE			
	R.B.C. (MILLION PER CU MM)	W.B.C. (PER CU MM)	HB (GM %)	HEMATOCRIT (%)
<i>Average Deviations for Dogs B, R, C and M</i>				
1	+0.04	+ 924	-0.25	+0.4
2	-0.32	+1,336	-0.35	-2.5
3	-0.50	+2,599	-0.65	-4.5
4	-0.74	+2,512	-0.60	-4.5
Sampling variation of each of these averages $\pm 2\sigma$	$\pm 0.54$	$\pm 3,282$	$\pm 1.24$	$\pm 2.56$
<i>Deviations for Dog Y</i>				
1	-0.36	-1,330	-1.00	0
2	+0.37	-1,330	-0.2	0
3	+0.27	+3,770	0	-2.0
4	+0.11	+10,180	-1.00	-3.0

*Body Weight and Nitrogen Balance*—All four animals (Dogs B, R, C, and M) receiving complete parenteral alimentation maintained or slightly increased body weight during the period of infusion. However, Dog Y which

received the same nutrient medium but without the fat lost 14 per cent of its initial weight. The nitrogen balance was slightly variable during the period of infusion. Some of the animals showed negative balance during the first

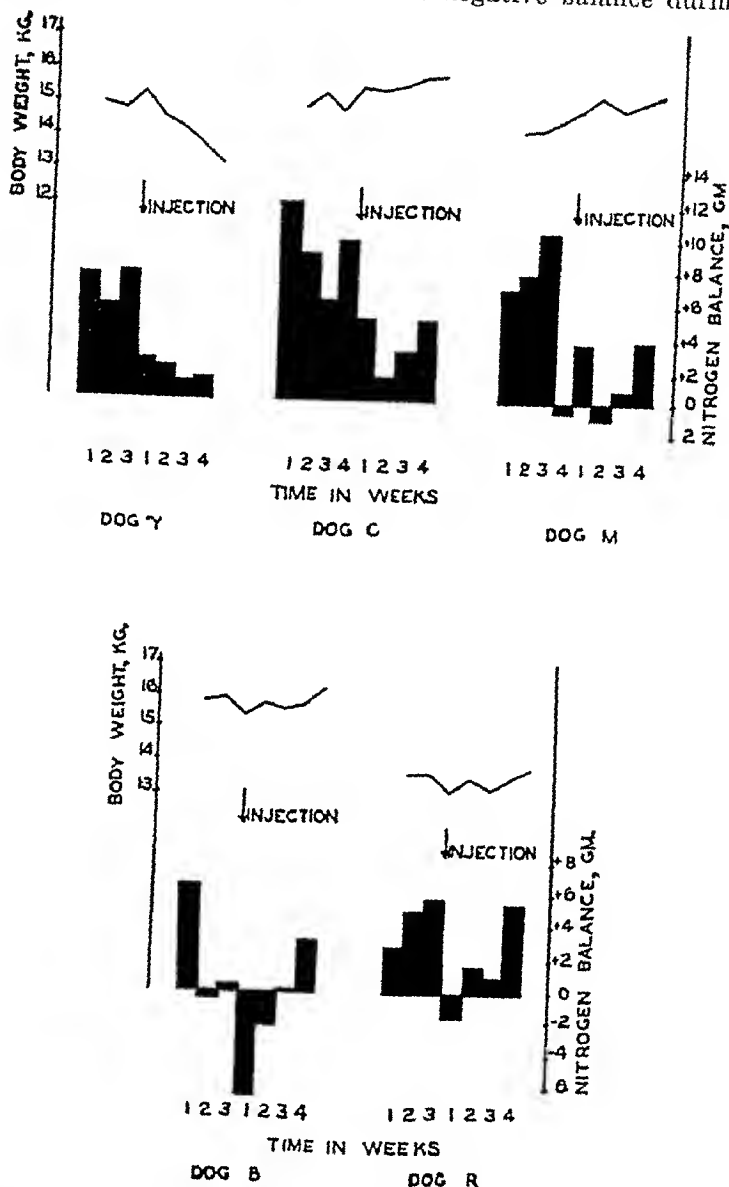


Fig 3—Showing the nitrogen balance and change in body weight during the complete parenteral alimentation period (Dog Y received no fat in the nutrient medium) similar to those during the control period

(Dog R) on second (Dog M) on both first and second (Dog B) weeks of infusion. However, negative balance also was observed on Dogs B and M during the second and fourth week respectively of the control period. The nitrogen balance of Dog Y during infusion was less positive than that during the control period. The results are shown in Fig 3.

"*Water Balance*"—During the control period the average "water balance" of the three day interval scheme for Dogs B, R, C, and M was +818 ml (ranging from 634 to 985 ml). The same animals averaged +1,447 ml (ranging from 969 to 1,938 ml) for the experimental period. The same change took place in Dog Y. The significance of these observations is doubtful since no account was taken of the insensible loss. There was probably no true water retention since there was no evidence of edema. The results of this study are presented in Table VII.

TABLE VII SUMMARY OF THE "WATER BALANCE"

DOG	PERIOD	NUMBER OF WEEKLY OBSERVATIONS	INTAKE (ML) 3 DAY			OUTPUT OF URINE (ML) 3 DAY (AVERAGE)	BALANCE (ML) 3 DAY (AVERAGE)
			WATER BY MOUTH (AVERAGE)	FLUID INJECTED (AVERAGE)	TOTAL (AVERAGE)		
B	Control	1	2,260	—	2,260	1,420	+ 840
R	Control	1	1,890	—	1,890	1,030	+ 860
C	Control	4	1,708	—	1,708	723	+ 985
M	Control	4	1,043	—	1,613	1,009	+ 034
Average for 4 dogs		10	1,755	—	1,755	938	+ 818
B	Exper	4	1,454	3,990	5,444	4,475	+ 969
R	Exper	4	1,860	3,420	5,280	4,235	+1,045
C	Exper	4	2,780	3,750	6,530	4,695	+1,835
M	Exper	4	2,285	3,660	5,945	4,008	+1,938
Average for 4 dogs		10	2,095	3,705	5,800	4,353	+1,447
Y	Control	3	2,070	—	2,070	1,240	+ 830
Y	Exper	4	3,453	2,550	6,003	4,690	+1,308

*Blood Chemistry and Liver Function*—There was no change in total plasma protein concentration. The plasma nonprotein nitrogen remained within the normal range throughout the infusion period except in the case of Dog M which showed a slight increase. However, there is no basis to assume that such change was due to renal damage incident to those infusions since the findings observed during the control period were high. The rose bengal clearance test was reduced in three of the four dogs (Dogs B, C, and M) receiving complete parenteral alimentation. Serum phosphatase was somewhat elevated in all the animals of this group. The animal (Dog Y) which received no fat demonstrated the same changes. However, the serum phosphatase determinations made just before the animals were sacrificed showed lowered or normal levels in all five dogs. These results are shown in Table VIII. Neither sugar nor albumin was detected in the urine throughout.

*Histologic Study*\*—Gross examination revealed no abnormalities at the end of the four week infusion period in both groups of animals (with or without the fat). Microscopically, Dogs B, R, C, and M, the animals receiving complete parenteral alimentation, showed a few stainable fat granules in some of the Kupffer cells. However, the parenchymal cells were fat free and normal. There was no fat observed in the liver of Dog Y. In the kidney sections of all five animals including the dog (Y) receiving no fat, there was positive fat stain in the cytoplasm of some of the distal convoluted tubules. The

\*Acknowledgment is made to Dr. James R. Dawson, Jr., Professor of Pathology, Vanderbilt University Medical School, for interpretation of the histologic sections.

glomeruli appeared normal and no fat was demonstrated. The sections of the lung of Dogs R, M, and Y showed normal structure and were fat free. There were three or four fat emboli observed in the lung section of Dog C, otherwise it was essentially normal. There was a very small ulcer which was in the acute stage seen in the section of the duodenum of Dog M, but no similar finding was seen in the sections of the other animals. Other sections revealed normal heart, pancreas, stomach, spleen, bone marrow, and adrenal and thyroid glands in all five experimental animals.

TABLE VIII EFFECT ON LIVER FUNCTION, TOTAL PLASMA, PROTEINS, AND PLASMA NONPROTEIN NITROGEN

DOG	PERIOD	WEEK	TOTAL PLASMA PROTEINS (GM %)	PLASMA N P N (MG %)	ROSE BENGAL CLEARANCE (%)	SERUM PHOSPHATASE* (ALKALINE)
B	Control	1	—	—	—	—
		2	7.27	24.5	104.0	9.45
		3	7.15	23.6	94.0	8.73
	Exper	1	—	—	—	—
		2	7.06	24.6	86.0	17.3
		3	—	—	—	—
R	Control	1	—	—	—	—
		2	6.83	21.1	123.0	5.58
		3	6.55	21.8	110.0	5.83
	Exper	1	—	—	—	—
		2	6.96	34.3	102.0	17.3
		3	—	—	—	—
C	Control	1	—	—	—	—
		2	7.6	20.0	93.0	4.9
		3	7.2	21.7	—	6.3
	Exper	1	—	—	—	—
		2	5.9	33.5	73.5	10.1
		3	—	—	—	—
M	Control	1	—	—	—	—
		2	6.8	56.4	97.0	4.3
		3	6.7	56.9	97.2	5.3
	Exper	1	—	—	—	—
		2	5.7	72.1	65.3	10.6
		3	—	—	—	—
Y	Control	1	—	—	—	—
		2	5.9	40.7	86.0	2.7
		3	6.2	42.0	62.2	3.6
	Exper	1	—	—	—	—
		2	6.3	32.9	69.7	4.6
		3	—	—	—	—

\*Bodansky units per 100 milliliters

†Just before animals were sacrificed

#### DISCUSSION

The animals used in the previous experiment<sup>7</sup> were injected while fastened on their sides on a canvas frame. It is obvious that the urinary bladder became very much distended since the animals were not accustomed to void in

such a position Hematuria resulted simply by rupture of the blood capillaries of the urinary bladder due to mechanical stretching. This was proved to be true by autopsy findings. By using the swivel device in this study, the animals were permitted to move at will. There was not the slightest difficulty encountered in urination or defecation and hematuria has never been observed.

Although the results showed a statistically significant fall of red cell count and hematocrit, it is reasonable to believe that the fat emulsion infused was not responsible for the slight change. In the first place, the observed average decrease for red cell count of Dogs B, R, C, and M was only -0.74 after the fourth week of complete parenteral alimentation with the fat. Second, Dog Y, the animal which received no fat, also demonstrated the reduction of hematocrit (Tables V and VI). Such small changes would not be surprising in view of the change of diet and degree of hydration. Finally, histologic examinations showed essentially normal bone marrow in all the experimental animals which would further support the belief.

The reduction of rose bengal clearance and elevation of serum phosphatase observed during complete parenteral alimentation indicate the impairment of liver function. However, the changes were demonstrated in all five dogs including the one which received no fat. The serum phosphatase determinations made just before the animals were sacrificed gave values which were at the control level (Table VIII). In view of this fact it seems reasonable to state that the signs of liver impairment were not caused by the intravenous administration of fat but rather were due to the complete parenteral alimentation in general since Dog Y which received no fat, demonstrated the same result. It is quite likely that during the infusion period the liver was under a heavy burden and had to work extremely hard in order to carry out the necessary processes for the utilization of the nutrients intravenously introduced. Furthermore the histologic examination of the liver showed normal parenchymal cells which again rules out the possibility of severe liver damage. The presence of a few fat granules in some of the Kupffer cells of the liver is probably as one might expect. However, it might be suggested that in clinical use if a patient's liver function is severely impaired, parenteral alimentation of any kind be given only after careful consideration.

The stainable fat found in the renal tubules is probably not the result of intravenous administration of fat. There was more stainable fat demonstrated in the tubules of Dog Y the animal receiving no fat, than in those of the fat infused animals. Albumin was never detected in the urine, and surprisingly enough glycosuria was never observed during the complete parenteral alimentation although the infusion rate of glucose was approximately 1.4 Gm per kilogram of body weight per hour. Woodyatt and associates<sup>11</sup> reported that 0.85 Gm per kilogram per hour (in 10 to 50 per cent solution) was the maximum speed of intravenous infusion of glucose without production of glycosuria. Lockhart and Elman<sup>12</sup> recently found that in human subjects 0.5 Gm per kilogram per hour was the maximum speed of infusion with

out production of glycosuria. This may be interpreted in part, at least, as being due to the following factors: (1) The addition of protein hydrolysate to the glucose solution increases the rate of utilization of glucose by the tissues as suggested by Lockhart and Elman<sup>12</sup>. (2) The addition of vitamin and mineral mixtures as carried out in the present study may further enhance the glucose-protein hydrolysate utilization. (3) The rate of glucose utilization may be faster in the dog than in man. Nevertheless, the absence of albuminuria and glycosuria demonstrates that there was no impairment of renal function which can be attributed to the complete parenteral alimentation.

The increase in urinary volume output during parenteral alimentation was simply due to the intravenous administration of a large quantity of fluid. The water intake by mouth of Dogs B, R, C, and M varied very little, with an average of 1,755 ml for the three-day interval scheme during the control period. Even during the experimental period, oral water intake of Dogs B and R was in this range. However, water intake by mouth of Dogs C and M was increased during the experimental period. The average water intake by mouth of Dog Y was 2,070 ml during the control period as compared with the average of 3,453 ml during the experimental period. The increase of water intake by mouth of Dogs C, M, and Y was probably due to the elevation of room temperature during the first part of the winter when the rooms were first heated. The water loss by channels other than urinary output, e.g., panting, was increased, leading to an increase of "water balance." However, it is not a true retention. The average "water balance" of Dogs B, R, C, and M during the control period was +818 milliliters. The average amounted to +1,447 ml during the experimental period for the same group of animals. The average increase of "water balance" for the three-day interval scheme was 629 ml which is only 209 ml per day. The average "water balance" of Dog Y, the animal receiving no fat, was also increased during the experimental period (+28 ml for three-day period) (Table VII). It is our belief that there was no disturbance in body fluid of different compartments during parenteral alimentation (with or without fat) in the present study.

The experiment of omitting fat from the diet for complete parenteral alimentation of Dog Y was planned in an attempt to obtain further evidence for the utilization of the intravenously administered fat. The animal (Dog Y) received inadequate caloric intake during the infusion period. It is assumed that if the infused fat is not utilized in the body then the results demonstrated by the two groups of animals (with or without the fat) should be similar. It is true that part of the intravenously administered protein hydrolysate is lost in the urine. Unfortunately, determination of amino nitrogen in the urine was not carried out in this study. However, it was probably not more than 10 per cent, since most investigators have shown it will not exceed this if the rate of infusion is not too rapid. The loss of amino nitrogen by means of the kidney might account for the difficulty in establishing nitrogen balance during parenteral alimentation. Nevertheless, the two groups of animals (with or without the fat) should appear similar as far as the nitrogen



loss is concerned. In view of these findings it is logical to believe that the injected fat is utilized in the body for energy. Dog Y lost 14 per cent of its initial body weight and appeared apathetic and emaciated while the other animals which received the fat were healthy, lively and in good spirits and maintained or increased their body weights (Fig 3). The gain in body weight of the animals receiving fat was not due to the deposition of the injected fat as adipose tissue as has been demonstrated by McKibbin and associates<sup>13</sup>. Moreover the dry, coarse hair and the epilation of the hind legs of Dog Y were probably due to a deficiency of the essential fatty acids as demonstrated by Evans and Burr<sup>14, 15, 16</sup> Burr and co workers,<sup>1, 18, 19</sup> and by Evans and Lepkovsky<sup>20, 21</sup> on rats, and by Hansen and Wiese<sup>2</sup> on dogs since low caloric intake (53 calories per kilogram per day) is probably not the only factor responsible for the general picture. Mann and associates<sup>3</sup> showed additional evidence of utilization of the intravenously injected fat in growing puppies. More recently Geyer and co workers<sup>4</sup> and Lerner and co workers<sup>5</sup> were able to demonstrate the presence of C<sup>14</sup> from the collected expired CO<sub>2</sub> from rats after intravenous injection of C<sup>14</sup> labeled triolein and tripalmitin fat emulsions. However further investigation should be carried out in this regard with adequate and inadequate caloric intake when fat is omitted in the parenteral basal ration to show the dispensableness of the fat.

The cause of the small ulcer observed in the duodenum section of Dog M is obscure. At any rate, there is no reason to suspect that it is related to complete parenteral alimentation. The presence of three or four fat emboli in the lung section of Dog C is certainly not significant insofar as the clinical picture is concerned since the animal was normal in all other aspects. The failure to show any pathologic changes in other histologic sections indicates that this emulsion could be used clinically with considerable confidence.

#### SUMMARY AND CONCLUSIONS

Dogs have been maintained for as long as four weeks on a diet administered exclusively by vein. The diet furnished proportional amounts of carbohydrate, protein, fat minerals, and vitamins. Thirty-four per cent of the caloric content of the diet was furnished by a special fat emulsion. The dogs remained healthy, lively, and in good spirits throughout and most of them gained weight. Extensive physiologic tests during the course of the experiments, and a complete histologic study following sacrifice, failed to reveal any significant abnormalities attributable to the procedure.

One dog was given the same treatment as the others except that the fat was omitted from the diet. It lost 14 per cent of its initial weight, appeared apathetic and emaciated and developed lesions suggestive of a fatty acid deficiency. The behavior of this dog gives considerable confidence to the belief that the injected fat in the other dogs was fully utilized and that its presence in the medium enhances the value.

It is concluded that this emulsion could be used clinically with considerable confidence, and that it would be very beneficial in cases where complete parenteral alimentation is necessary.

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# THE NUTRITIVE VALUE OF INTRAVENOUSLY ADMINISTERED HYDROLYZED HUMAN SERUM ALBUMIN IN MAN

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HUMAN serum albumin serves as a protein nutrient for human subjects when administered in the intact form by mouth or by vein<sup>1 2 3</sup> The present study was undertaken to determine its nutrient value when administered intravenously in the hydrolyzed form as amino acids The results indicate that the parenterally administered hydrolyzed albumin, supplemented with l-tryptophane, was nutritionally adequate for a slightly undernourished subject and was metabolized and excreted in a manner similar to that of other hydrolysates

## MATERIALS AND METHODS

The subject selected for the study was a 43 year old man who was admitted to the hospital ten days previously for acute alcoholism His general health in the past had been excellent, although during his intermittent alcoholic sprees he drank heavily and ate poorly and had recently lost 10 pounds in weight The physical examination and laboratory findings, including tests of liver function were entirely within the limits of normal The subject was asymptomatic throughout the twenty third day period of study while hospitalized on the Thorndike metabolic ward

A 50 Gm protein diet was used in the first period and supplied 3,000 calories daily It contained the foods ordinarily eaten in a well balanced, nutritious diet The nitrogen content was calculated from standard food tables In the second period a protein free diet was used which supplied 3 500 calories but only 0.4 Gm of nitrogen daily as determined by macro Kjeldahl analysis, the diet has been described previously<sup>2</sup>

The hydrolyzed albumin solution was prepared by the complete acid hydrolysis of human serum albumin and was supplemented with l-tryptophane† The composition of the solution and the quantity of amino acids infused are given in Table I Previously reported values for the amino acid content of casein<sup>4</sup> and of human serum albumin<sup>5 6</sup> are listed for comparison One liter of the 7 per cent solution was administered intravenously once daily, together with 100 cc of 50 per cent dextrose The injection was made one hour after a 1,000 calorie breakfast in order to achieve maximum utilization of the infused amino acids by the simultaneous provision of adequate carbohydrate and protein<sup>7</sup>

The daily urine and pooled stool nitrogen analyses were determined by the standard micro or macro Kjeldahl methods The urinary alpha amino nitrogen was determined by the gasometric ninhydrin method described by Van Slyke MacFadyen, and Hamilton<sup>8</sup> The amino acid analyses of the urine and of the albumin hydrolysate solution were by the microbiologic method of Stokes and co workers<sup>4</sup>

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†The human serum albumin was processed and supplied by the Medical Research Division Sharp & Dohme Inc Glenolden Pa from blood collected by the American National Red Cross from voluntary donors The hydrolysate was specially prepared for this investigation by acid hydrolysis of the albumin which had been rejected for clinical use because of pyrogen content The hydrolysis preparation and analysis of the pyrogen free 7 per cent solution were done under the direction of Dr E E Howe Merck & Co Inc Rahway N J The authors express their thanks to the individuals and organizations concerned

TABLE I AMINO ACID COMPOSITION OF THE HYDROLYZED HUMAN SERUM ALBUMIN SOLUTION

AMINO ACID	HYDROLYZED HUMAN SERUM ALBUMIN*		HUMAN SERUM ALBUMIN† (GM/100 GM)	CASEIN‡ (GM/100 GM)
	GM /INJECTION	GM /100 GM		
Arginine	4.5	6.5	6.2	3.9
Histidine	2.4	3.5	3.5	2.8
Isoleucine	1.1	1.6	1.7	5.6
Leucine	8.9	12.8	11.9	9.9
Lysine	7.3	10.5	5.8	7.7
Methionine	0.9	1.3	1.3	2.6
Phenylalanine	5.4	7.8	—	5.9
Threonine	4.0	5.8	5.0	4.2
Tryptophane	1.2	1.7 (Added)	0.19	1.1
Valine	4.7	6.8	—	6.7

\*Determinations by microbiologic assay. †Prepared for intravenous administration as a 7 per cent solution and each 1000 c.c. infusion supplies (by analysis) 69 Gm. of amino acids 11.1 Gm. of total nitrogen and 8.7 Gm. of alpha amino nitrogen.

†Values of Brand, Kassell and Saidel\* except isoleucine which is the value of Hegsted and Stare\*.

‡Microbiologic values of Stokes and co-workers\*.

## RESULTS

The solution of hydrolyzed human serum albumin was tolerated well clinically when administered intravenously over a period of approximately two and one-half hours. An attempt to administer the solution more rapidly was followed, on two occasions, by flushing and warmth of the face and upper neck together with mild nausea. Thrombosis of the infusion vein did not occur.

The nitrogen balance data for the subject are summarized in Table II. Nitrogen equilibrium was maintained during an initial five day control period while the subject received the basal 50 Gm. protein diet. Supplementation of this diet by the addition of 70 Gm. of hydrolyzed human serum albumin (1,000 c.c. of the 7 per cent solution) intravenously daily for five days resulted in a

TABLE II NITROGEN BALANCE DATA FOR SUBJECT GIVEN HYDROLYZED HUMAN SERUM ALBUMIN INTRAVENOUSLY

PERIOD OF STUDY (DAYS) AND CONDITION OF STUDY	NITROGEN INTAKE (GM/DAY)		NITROGEN OUTPUT (GM/DAY)		NITROGEN BALANCE (GM/DAY)
	ORAL	I V	URINE	STOOL	
1-6 Fore control	8.1	0.0	6.7	1.2	+0.2
6-11 Hyd. albumin, I V	8.1	11.1	11.8	1.2	+6.2
11-17 After control	8.1	0.0	6.4	1.2	+0.5
17-24 Hyd. albumin, I V	0.4	11.1	10.2	0.4	+0.9

sustained positive nitrogen balance averaging +6.2 Gm. daily. The nitrogen balance during a six-day after-control period was comparable with that observed initially, and equilibrium again was attained by the 50 Gm. of dietary protein. During the final seven days of study, the subject received the diet adequate in calories but essentially devoid of protein and 1 liter of the 7 per cent solution of hydrolyzed albumin (70 Gm. of amino acids) intravenously daily as the sole source of nitrogen. Nitrogen equilibrium and extreme constancy of the urinary

nitrogen excretion were observed. The nitrogen balance averaged +0.9 Gm daily. The subject's body weight remained constant throughout the twenty-three day study period.

The excretion of alpha amino nitrogen and of the ten "essential" amino acids in the urine during each of these periods of study is tabulated in Table III. While receiving 50 Gm of dietary protein the subject excreted comparable quantities of amino acids during both the fore and after control periods. The urinary excretion of amino acids was considerably increased when hydrolyzed albumin (70 Gm of amino acids) was administered intravenously, whether as a supplement to the 50 Gm protein diet or as the sole source of nitrogen. While receiving the parenterally injected hydrolyzed albumin as supplemental protein, the subject showed a loss by excretion in the urine of from 0.2 to 14.2 per cent of the individual "essential" amino acids infused, of 2.7 per cent of the total of the ten "essential" amino acids injected and of 11.9 per cent of the alpha amino nitrogen administered. These losses were somewhat less when the hydrolyzed albumin was administered intravenously as the sole source of nitrogen. In this instance, the maximum loss of any individual "essential" amino acid infused was 8.4 per cent and averaged

TABLE III. EXCRETION OF AMINO ACIDS IN THE URINE FOLLOWING INFUSIONS IN HYDROLIZED HUMAN SERUM ALBUMIN

AMINO ACID	CONSTANT 50 GM PROTEIN DIET				PROTEIN FREE DIET			
	CONTROL (MG / DAY)	PLUS 70 GM HYD ALBUMIN, I V			CONTROL (MG / DAY)	PLUS 70 GM HYD ALBUMIN I V		
		MG / DAY	EXCESS (MG / DAY)	PER CENT EX- CRETED†		MG / DAY	EXCESS (MG / DAY*)	PER CENT EX- CRETED†
Arginine	10.0	39.1	29.1	0.7	10.0	48.4	38.4	0.9
Histidine	12.3	4.63	3.40	14.2	13.0	33.1	20.1	8.4
Isoleucine	5.5	7.7	1.9	0.2	5.5	18.4	12.9	1.2
Leucine	11.5	43.0	32.1	0.4	11.5	53.2	41.7	0.5
Lysine	19.7	39.7	37.7	5.2	19.5	14.0	12.1	1.7
Methionine	3.7	13.6	9.9	1.1	3.3	10.5	7.2	0.8
Phenylalanine	15.1	58.7	43.6	0.8	15.1	78.3	63.2	1.2
Threonine	14.0	22.6	21.2	5.3	13.3	18.6	17.3	4.3
Tryptophane	12.3	16.4	4.1	0.3	11.8	10.7	0.0	0.0
Valine	5.4	42.5	37.1	0.8	6.7	51.1	44.4	0.9
Total 10 'essential' amino acids	220	1307	1087	2.7	227	928	701	1.7
Alpha amino nitrogen	185	1221	1036	11.9	186	678	492	5.7

\*Milligrams of amino acid or of alpha amino nitrogen in excess of that excreted during the preceding control period (see text footnote).

†Per cent of administered amino acid or of administered alpha amino nitrogen (Table I) excreted in urine.

The amino acid excretion values listed in the last two columns of Table III are in excess of the amount of alpha amino nitrogen and of individual amino acids excreted during the preceding 50 Gm protein diet period. Since the subject received the hydrolyzed albumin as the sole source of nitrogen during this final period these values should be in excess of the amount of amino acids excreted while he was receiving the diet devoid of protein. This was not determined. However, since the minimum endogenous amino acid excretion is increased only slightly by the ingestion of protein, the error incurred in the calculation of these values is small, particularly in view of the considerably increased excretion of amino acids following infusions of protein hydrolyzates.

17 per cent for the total of the ten "essential" amino acids administered, while only 5.7 per cent of the alpha amino nitrogen injected was excreted in the urine. In each instance, however, the subject excreted considerably greater percentages of administered histidine and threonine, and to a lesser extent lysine, than of the other "essential" amino acids in the infusion mixture.

During the periods while hydrolyzed albumin was administered intravenously the urinary excretion of total nitrogen, of alpha amino nitrogen, and of each of the ten "essential" amino acids promptly increased and remained constant throughout its administration. The day the hydrolysate infusions were discontinued, these values immediately returned to those of the preceding control period. Thus, the data presented in Tables II and III and averaged for each period of study are valid representations of the changes that occurred daily.

#### DISCUSSION

This study demonstrates that a complete acid hydrolysate of human serum albumin, supplemented with *L*-tryptophane, can maintain weight and nitrogen balance for short periods of time in man when administered intravenously (70 Gm of amino acids daily) as the sole source of food nitrogen. Furthermore, approximately half of that injected parenterally into the subject as a supplement to a 50 Gm protein diet was retained and presumably formed extra body tissue. The solution employed, therefore, contained all the essential amino acids required by man for maintenance of nitrogen balance and weight, and for tissue protein repletion when administered in adequate amounts by vein for short periods of time. In these regards, intravenously administered hydrolyzed human serum albumin was metabolized and utilized in a manner similar to hydrolysates prepared from other protein sources.

The values for the amino acid composition of hydrolyzed human serum albumin reported in this paper and determined by microbiologic assay agree quite closely with those previously reported (Table I).<sup>4, 5</sup> The lysine content was higher than found before, while the phenylalanine and valine contents (7.8 and 6.8 Gm per 100 Gm of albumin respectively) have not been published previously.

The low isoleucine and tryptophane content of albumin, particularly when compared with casein (Table I), previously occasioned concern over the protein's nutritional adequacy, especially since albumin was found incapable of supporting growth when fed to young rats until extra quantities of these amino acids were provided.<sup>6</sup> However, no supplementation was required for maintenance of nitrogen balance in the adult dog.<sup>6</sup> Previous studies in this laboratory demonstrated that adequate quantities of orally ingested human serum albumin, not supplemented with isoleucine or tryptophane maintained nitrogen balance and weight for short periods of time in a normal subject.<sup>2</sup> Furthermore, the provision of additional isoleucine and tryptophane failed to alter nitrogen excretion and balance whether albumin was given by mouth in amounts insufficient to maintain nitrogen equilibrium or by vein in amounts

adequate to achieve positive nitrogen balance. It also has been demonstrated that unsupplemented albumin serves as a protein nutrient when administered in the intact form intravenously.<sup>1, 2</sup> Finally, the nutritional adequacy of albumin administered intravenously in the hydrolyzed form as amino acids supplemented with L-tryptophane was demonstrated in the present study. Since tryptophane is destroyed upon acid hydrolysis it was necessary to supplement the solution with this "essential" amino acid although considerably more was added (12 Gm. per liter of 7 per cent solution) than is present in the native protein (0.19 Gm. per 100 Gm. of albumin). Since albumin containing only 0.19 per cent tryptophane is nutritionally adequate when administered in the intact form by mouth or by vein it would be unlikely that it is inadequate when administered intravenously in the hydrolyzed form as amino acids. Under these conditions however, somewhat larger quantities of the hydrolysate might have to be provided since the quantitative requirement of hydrolyzed protein administered intravenously is probably greater than that of an equivalent quantity given orally.<sup>10, 11</sup>

The values for alpha amino nitrogen and for the ten "essential" amino acids excreted in the urine by the subject while receiving the 50 Gm. protein diet (Table II) are in general agreement with those reported previously from this laboratory<sup>9, 12</sup> and by others employing microbiologic methods.<sup>13, 14</sup> These values were considerably increased when hydrolyzed albumin was administered

TABLE IV. COMPARISON OF AMINO ACID EXCRETION FOLLOWING INFUSIONS OF HYDROLYZED ALBUMIN AND HYDROLYZED CASEIN

AMINO ACID	ADMINISTERED				EXCRETED					
	GM./INFUSION		% OF TOTAL		MG./24 HR.		% OF TOTAL		% OF THAT GIVEN	
	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN	HYD. ALB.	HYD. CASEIN
Arginine	45	25	11.1	9.1	29.1	8.7	2.7	1.2	0.7	0.3
Histidine	2.4	1.6	6.0	5.1	3.40	2.06	31.3	28.8	14.2	12.9
Isoleucine	1.1	7.8	2.7	12.6	1.9	9.1	0.2	1.3	0.2	0.2
Leucine	8.9	7.8	22.0	20.7	32.1	45.7	2.9	6.4	0.4	0.6
Lysine	7.3	4.2	18.1	13.9	37.7	14.9	34.7	20.8	5.2	3.5
Methionine	0.9	3.2	2.2	10.6	9.9	34.4	0.9	4.8	1.1	1.1
Phenylalanine	5.4	2.7	13.4	9.9	47.6	40.1	4.0	6.0	0.8	1.6
Threonine	4.0	1.1	9.9	3.0	21.2	15.5	19.5	21.6	5.3	14.1
Tryptophane	1.2	0.45	3.0	1.5	4.1	22.7	0.4	3.2	0.3	5.7
Valine	4.7	3.1	11.6	10.2	37.1	42.1	3.4	5.9	0.8	1.4
Total	40.4	30.4	100.0	100.0	103.7	71.6	100.0	100.0	2.1	2.3

Averaged values following infusions of 500 cc. of a 10 per cent solution of hydrolyzed casein to two normal subjects in approximately one hour.<sup>12</sup>

intravenously. Since albumin and casein differ in regard to their amino acid composition (Table I), the amino acid excretion following infusions of hydrolyzed albumin was compared with that following infusions of a hydrolysate of casein previously reported from this laboratory.<sup>1</sup> These data are shown in Table IV. Despite differences in the amino acid composition of these two solutions, quite similar percentages of each administered amino acid were excreted in the urine and the postinfusion pattern of the "essential" amino

acids was similar whether hydrolyzed albumin or hydrolyzed casein was injected. The individual amino acids, however, were not excreted in the same proportion as administered in either hydrolysate, so that considerably greater percentages of administered histidine and threonine, and to a lesser extent lysine, were excreted than of the other "essential" amino acids. Despite the difficulty in interpreting these observations, as discussed previously,<sup>12</sup> the similarity in the renal excretion of amino acids following infusions of hydrolyzed albumin and of hydrolyzed casein is apparent.

#### SUMMARY AND CONCLUSIONS

1 One liter of a 7 per cent solution of hydrolyzed human serum albumin, prepared by the complete acid hydrolysis of albumin and supplemented with *L*-tryptophane, was administered intravenously daily to a slightly undernourished subject for five days as a supplement to a 50 Gm protein diet, and for seven days as the sole source of food nitrogen.

2 Nitrogen equilibrium was maintained when hydrolyzed albumin (70 Gm of amino acids) was injected intravenously as the sole source of nitrogen, while a sustained positive nitrogen balance was achieved during its administration as supplemental protein. The subject's body weight remained constant throughout the study.

3 The excretion of alpha amino nitrogen and of the ten "essential" amino acids in the urine following the intravenous administration of the hydrolyzed albumin solution was similar to that previously observed following infusions of hydrolyzed casein.

4 It is concluded that parenterally administered hydrolyzed human serum albumin, supplemented with *L*-tryptophane, is nutritionally adequate for man and is metabolized and excreted in a manner similar to that of hydrolysates prepared from other protein sources.

The authors wish to thank Alice Ballou, Ellen Doyle, and Elaine Hushberg for technical assistance, and Kathleen Clinton for preparing and calculating the diets used.

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# ABSORPTION OF UNEMULSIFIED AND EMULSIFIED VITAMIN A IN SPRUE

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## INTRODUCTION

A CHIEF result of the sprue syndrome is the impaired absorptive power of the small intestine. Among the prominent features of the syndrome are deficiency of hematopoietic essentials, fat-soluble vitamins, minerals, and protein. Diminished absorption of ingested foods, especially fats and fat-soluble substances, is an important cause for the development of the many specific deficiencies. Even though therapy alleviates most of the symptoms, restores the weight, the blood picture, glucose absorption, and gastric acid secretion to reasonably normal levels, there still remains in a number of patients the problem of persistent steatorrhea. Much of the disability in sprue patients can be attributed to steatorrhea. With the steatorrhea, there is impaired absorption of vitamin A, vitamin K, and calcium.

Vitamin A deficiency does not always manifest itself by clinical evidence but may be demonstrated only with biochemical methods. The investigations reported here are concerned only with the aspect of vitamin A deficiency in sprue. A report of numerous other studies conducted on these patients will appear elsewhere.

Because previous investigators<sup>1</sup> had shown that vitamin A absorption was markedly augmented in children with steatorrhea when fed emulsified materials, it was decided to conduct similar studies on the comparable effect of feeding unemulsified and emulsified vitamin A preparations to adult patients with sprue.

## MATERIALS AND METHOD OF STUDY

The procedures for doing plasma vitamin A determinations were similar to those previously described.<sup>2,3</sup> The vitamin A levels herein reported are listed in micrograms. One microgram of vitamin A represents approximately 25 I U. For the unemulsified vitamin A material, oleum percomorphum was used. The vitamin A content of this fish liver oil is rated at 60,000 I U per gram and is largely in the form of esters. The emulsified vitamin A material used was a special preparation. The emulsion was composed of 15 per cent vitamin A acetate, 15 per cent Tween 20,\* and 70 per cent water. This emulsion contained 100,000 I U of vitamin A per gram. Determinations were made on plasma specimens taken fasting and at intervals of three, five, and seven hours after the feeding of a test dose of 200,000 µg of vitamin A material. The peak rise in vitamin A occurred during the three to five hour period and fell to lower levels at seven hours. Therefore, only the fasting and the three and the five hour levels are included in this report to show the maximum rise. The test dose of vitamin A material used in all these experiments was given to the patients after an overnight fasting period. Milk was used as a vehicle for the materials followed by feeding a normal breakfast.

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\*The Tweens (Atlas Powder Co., Wilmington, Del.) are very effective emulsifying agents and are nontoxic when fed to human beings.

Three groups of patients were used for obtaining vitamin A absorption curves. For the first group of absorption curves eight normal control patients were used. The next group of absorption curves was obtained from eight patients with sprue in relapse. Finally, a third group of observation curves was obtained on six patients who had had sprue but in whom there were no detectable clinical nor laboratory signs of activity and therefore were classified as having sprue in remission.

Tolerance curves on the patients were first obtained with unemulsified vitamin A ester in fish liver oil. Within a week of this original study another tolerance curve was obtained on the same patients but this time with the feeding of emulsified vitamin A acetate preparation. To each of the three groups of patients was administered the unemulsified vitamin A ester in fish liver oil. To the group of normal patients and to those with sprue in relapse was administered the vitamin A acetate emulsion composed of 15 per cent vitamin A acetate, 15 per cent Tween 20, and 70 per cent water. The test dose in terms of micrograms of vitamin A used in each preparation was the same.

## RESULTS

The vitamin A levels as determined by the procedures referred to are compared and summarized in Tables I and II.

TABLE I. COMPARISON OF VITAMIN A ABSORPTION TEST IN NORMAL CONTROLS AND IN PATIENTS WITH SPRUE  
(Oleum Percomorphum 200,000 µg vitamin A per patient test dose)

CASE	VITAMIN A VALUES IN µg/100 ML OF PLASMA			
	FASTING	3 HR	5 HR	MAX RISE
<i>Normal Controls</i>				
8 normal controls	Range 40-80 Av 60	11-280 195	240-505 374	314
<i>Sprue in Relapse</i>				
1	58	50	46	00
2	29	31	44	15
3	48	64	100	52
4	65	75	73	10
5	71	134	115	63
6	39	46	72	33
7	10	22	25	13
8	43	49	73	30
<i>Sprue in Remission</i>				
9	51	72	282	231
10	52	58	192	140
11	86	214	405	319
12	64	119	420	356
13	67	91	376	309
14	58	198	356	298

In normal adult patients the feeding of unemulsified vitamin A ester in fish liver oil produced significant rises in plasma vitamin A. When the same group of normal patients was given emulsified vitamin A acetate in Tween a similar but higher peak level in plasma vitamin A was produced. The emulsified material accelerated absorption in only one instance producing a peak rise at three hours compared to a peak reached in five hours in the remainder of this group.

Adult patients with active sprue who were requiring maintenance dosage of folic acid or liver therapy and all of whom continued to show steatorrhea were fed unemulsified vitamin A ester in fish liver oil. Very poor absorption

TABLE II COMPARISON OF VITAMIN A ABSORPTION TEST IN NORMAL CONTROLS AND IN PATIENTS WITH SPRUE UNEMULSIFIED AND EMULSIFIED MATERIALS

(A, Oleum Percomorphum, 200,000  $\mu$ g vitamin A per patient, test dose, B, Vitamin A Acetate emulsion, 200,000  $\mu$ g vitamin A per patient test dose)

CASE AND MATERIAL	VITAMIN A VALUES IN $\mu$ G/100 ML OF PLASMA			
	FASTING	3 HR	5 HR	MAX RISE
<i>Normal Controls</i>				
5 normal controls	Range 40 80	250 640	530 980	
B	Av 60	410	755	695
<i>Sprue in Relapse</i>				
1 A	58	50	46	00
B	55	432	485	430
2 A	29	31	44	15
B	27	290	340	313
3 A	48	64	100	52
B	57	300	480	427
4 A	65	75	73	10
B	63	710	942	879
5 A	71	134	113	63
B	63	620	946	883
6 A	39	40	72	33
B	56	380	695	639
7 A	10	22	23	13
B	9	240	495	486
8 A	43	49	73	30
B	33	70	115	82

resulted, as may be seen in Table I, the maximum rise of vitamin A being quite low in every patient. However, when preparations of emulsified vitamin A acetate in Tween were fed to this same group, a very marked increase in plasma vitamin A occurred of about the same degree as seen in normal controls. Only one patient, Case 8 in Table II, repeatedly proved an exception and showed very little rise in plasma vitamin A when given emulsified vitamin A acetate in Tween.

When the group of patients classified as having *sprue in remission* were fed unemulsified vitamin A ester in fish liver oil, the results as seen in Table I were, with one exception, fairly comparable to those obtained in the normal controls. After two members of this group were found to have normal absorption curves when fed the emulsified vitamin A material, it was decided not to expend the small supply of this preparation on the whole of the group.

#### DISCUSSION

Under normal physiologic conditions the absorption curves on adults demonstrate that the tested materials were well absorbed and that the peak levels of plasma vitamin A were somewhat higher following the use of emulsified preparations. Of chief interest were the striking differences in results obtained in patients with *sprue in relapse*. The marked improvement in vitamin A absorption in these patients, with one exception, was dependent upon the use of emulsified materials. Such emulsions, it has been determined,<sup>1</sup> contain only droplets varying in size from 0.5 to 10.0 microns. It has been postulated<sup>4</sup> that the degree of the dispersion of fat is perhaps mainly responsible for absorption. Finely dispersed particles result from a suitable emulsifying system. The emulsifying agent, Tween 20, apparently produced particles of appropriate size for enhanced absorption.

Fish liver oil contains vitamin A largely in the form of esters. Therefore, it is thought that the mechanism for its digestion is similar to that of other neutral fats and requires lipolysis. In patients with sprue there is no evidence of defective lipolysis. However, it is to be noted that fish liver oil is *unemulsified* material. Therefore, the hypothesis presented by one investigator that absorption is mainly dependent upon fine particle size seems to be further supported by the data herein reported.

The observations on patients with sprue in remission demonstrate that they absorbed the unemulsified material with one exception equally as well as normal controls. This would indicate a return to a normal physiologic status of the small intestinal tract. Further evidence of this was found in the normal clinical condition, normal radiologic pattern of the small intestines, and normal quantitative fecal fat.

It should be noted that we do not know the degree of absorption which might result from feeding vitamin A acetate alone. Would this form of vitamin A be as well absorbed without the addition of the emulsifying agent used in these experiments? Since it was impossible to obtain the necessary amounts of vitamin A in this pure form we cannot answer this question. However it has been reported that patients with steatorrhea who failed to absorb vitamin A esters in fish liver oil did show a marked increase in vitamin A absorption when polyoxyethylene sorbitan monooleate 'Tween 80' was added to the test dose of the same material.

The implications of these data would seem to be of great practical importance in the management of steatorrhea in sprue. These patients present a difficult problem in dietary fat control. It is probable that their poor absorption of vitamin A, as well as fats results from a defective emulsifying system in the small intestinal tract. Therefore the ideal diet should probably be one containing vitamin A and fats in emulsified form or else more practical the addition of an emulsifying agent to the diet.

#### SUMMARY

The feeding of emulsified vitamin A to normal adults resulted in a somewhat higher elevation of plasma vitamin A than did the feeding of unemulsified vitamin A material.

The patients with sprue in remission absorbed unemulsified vitamin A to a normal degree with only one exception.

Patients with active sprue showed very poor absorption when fed unemulsified vitamin A. When these same patients were fed emulsified material, the absorption of vitamin A was increased to approximately normal with one exception.

It is suggested from these results that patients with active sprue require the addition of an emulsifying agent to their dietary fat and fat soluble substances to promote absorption.

For the preparation and generous supply of emulsion of vitamin A acetate with Tween 20, we are greatly indebted to Dr. A. E. Osterberg of Abbott Research Laboratories, Durham, N. C. For performing the blood examinations we are indebted to Mrs. G. Stenhouse and Mrs. J. D. Moody.

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## LABORATORY METHODS

### A NEW TABLET TEST FOR URINARY BILIRUBIN

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THE need for a practical solution of the clinical problem of detecting small amounts of urinary bilirubin has received added emphasis with the increased incidence of liver disease during World War II<sup>1</sup>. Until recently we have had no simple sensitive and reliable bilirubin test which could be used routinely by the physician for the detection of bile in the urine and which could be employed in the armed forces and industry for mass examinations to detect latent liver disease or follow its course. The standard methods for the detection of bilirubin in the urine such as the foam, nitric acid, and iodine tests are relatively insensitive and are difficult to evaluate when pigments other than bilirubin are present in the urine<sup>2</sup>. The methylene blue test<sup>3,4</sup> although simple and useful as a screening or follow up test, is nonspecific and may give falsely positive results with urine containing yellow pigments such as riboflavin, penicillin or Atabrine. It is also less sensitive than other bile tests routinely employed<sup>5</sup>. Tests depending upon concentration of the bile pigment either by precipitation and adsorption of the pigment by barium chloride or calcium chloride or upon adsorption by talc are the most sensitive tests<sup>6</sup>. Such tests as the Godfried<sup>10</sup> diazo and Harrison<sup>11</sup> and Naumann<sup>12</sup> spot tests are relatively time consuming. Watson's<sup>4</sup> barium strip modification of the Harrison test has best met the requirements of sensitivity, simplicity, and applicability to mass and serial usage. With this test, higher concentrations of bilirubin give a definite green color. However, at low concentrations of bilirubin (below 0.1 mg per 100 cc) the color becomes blurred and indistinct and we have found the test often to be negative (Table I).

The object of this paper is to describe a simple yet sensitive test which is applicable to clinical work by the physician in everyday practice and to mass screening and serial testing. It is a tablet test, which can be prepared very simply and inexpensively. This test makes use of the principles of concentration of biliary pigment as in the Naumann and Harrison spot tests and subsequent oxidation with resultant color change of the bile pigment by Fouchet's reagent\*. Concentration of bilirubin pigment in the Naumann

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Fouchet's reagent consists of

Trichloroacetic acid	Gm
Distilled water	100 c.c.
10 per cent ferric chloride solution	10 c.c.

TABLE I SENSITIVITY COMPARISON OF THE FRIEDEL TEST AND TABLET TEST FOR BILIRUBINURIA

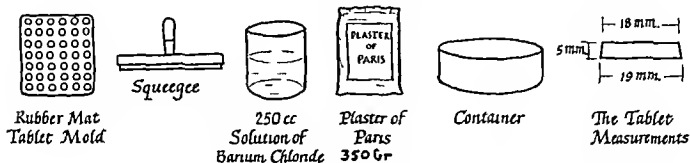
Urine A											
Urine dilution	0	1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9	1/10	Drops of Urine
Concentration* bilirubin (mg %)	15.7	7.85	5.14	3.87	2.58	1.94	1.57	1.19	0.95	0.76	1/100
Filter paper test	4+	4+	2+	2+	2+	1+	1+	1+	1+	0	0
Tablet test	4+	4+	2+	2+	2+	1+	1+	1+	1+	0	0
Urine B											
Urine dilution	0	1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9	1/10	Drops of Urine
Concentration* bilirubin (mg %)	2.4	1.2	0.8	0.6	0.48	0.4	0.34	0.28	0.23	0.19	1/100
Filter paper test	3+	1+	1+	1+	1+	1+	1+	1+	1+	0	0
Tablet test	3+	2+	1+	1+	1+	1+	1+	1+	1+	0	0
Urine C											
Urine dilution	0	1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9	1/10	Drops of Urine
Concentration* bilirubin (mg %)	1.5	0.75	0.5	0.375	0.3	0.25	0.21	0.17	0.14	0.11	1/100
Filter paper test	2+	1+	1+	1+	1+	1+	1+	1+	1+	0	0
Tablet test	2+	2+	1+	1+	1+	1+	1+	1+	1+	0	0
Urine D											
Urine dilution	0	1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9	1/10	Drops of Urine
Concentration* bilirubin (mg %)	6.5	3.25	2.17	1.63	1.3	1.08	0.94	0.8	0.68	0.58	1/100
Filter paper test	3+	2+	2+	2+	2+	1+	1+	1+	1+	0	0
Tablet test	3+	2+	2+	2+	2+	1+	1+	1+	1+	0	0
Urine E											
Urine dilution	0	1/2	1/3	1/4	1/5	1/6	1/7	1/8	1/9	1/10	Drops of Urine
Concentration* bilirubin (mg %)	1.2	0.6	0.4	0.3	0.24	0.2	0.16	0.13	0.11	0.09	1/100
Filter paper test	2+	1+	1+	1+	1+	1+	1+	1+	1+	0	0
Tablet test	2+	1+	1+	1+	1+	1+	1+	1+	1+	0	0

\*Concentration of urinary bilirubin determined by Gibson's method  
 †Watson's modification of Harrison's spot test

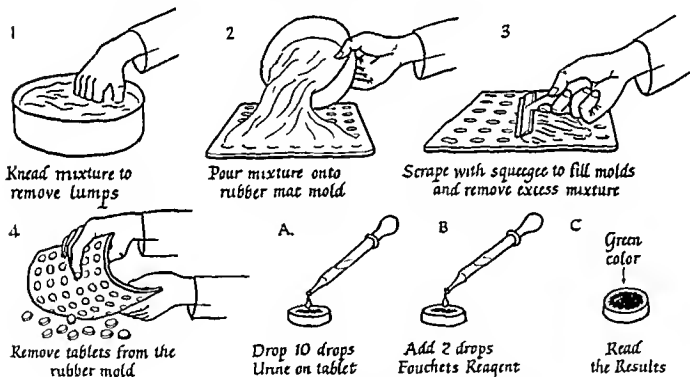


test is achieved by adsorption to a precipitated emulsion of talc. The Harrison spot test utilizes barium chloride as the adsorbing agent for bilirubin. The tablets used in this test concentrate bilirubin by utilizing the processes of filtration, adsorption by gypsum particles, and barium chloride adsorption. This test is thus a modification of both the Naumann and Harrison spot tests.

## MATERIALS



## PROCEDURE IN MAKING TABLET AND TEST



## PROCEDURE OF TEST

Fig 1

## DESCRIPTION OF METHOD

**A Preparation of Tablets (Fig 1 14)**—Three hundred fifty grams of ordinary commercial plaster of Paris (not dental plaster) are mixed in a container with 250 c.c. of a saturated solution of barium chloride. The resultant mix is then stirred and kneaded manually to remove any lumpy particles and to produce a homogeneous plaster mix which can be poured. This should be done in less than ninety seconds or the plaster will set in the container. It is then poured into a suitable rubber mold with approximately 250 indentations, each of which is 18 mm in diameter at the bottom, 19 mm at the top, and 5 mm deep. This is the size of the finished tablets. Before pouring the plaster, the rubber mat should be moistened by placing it under a running faucet or dipping it in water. The mat is inverted to get rid of excess water and the plaster is then poured onto the moistened mat. A squeegee (used to clean windows) is then drawn over the surface of the mat several times, distributing the wet plaster evenly in the indentations. The plaster is allowed to become firm and after

fifteen minutes the mat is inverted and the firm but still moist tablets will drop out. These are then dried in an oven and are ready for use at any time thereafter. The entire procedure of preparing the tablets until they are ready for drying should not take more than twenty minutes.

With the described proportions of plaster of Paris and barium chloride solution, the tablets should be of the proper consistency and should give optimal results. The proportion of 1 1/4 Gm. of plaster of Paris for every 1 c.c. of saturated barium chloride may have to be altered somewhat, depending upon the brand of plaster of Paris used. If the resultant tablets are too hard and do not absorb the urine quickly enough, the proportion of plaster of Paris to the saturated barium chloride may be decreased. Ordinary tap water instead of the saturated barium chloride solution may be used in the preparation of the tablet. However, tablets prepared in this manner do not absorb as much bilirubin, making the test less sensitive. The rubber mold we have used is an ordinary commercial door mat,\* 1 ft. by 2 ft., containing 500 indentations. The upper ridged surface is made smooth with sandpaper and the mat is cut in two, one half of this mat being used because it is easier to work with a smaller mold. An improved mat could be prepared with indentations of proper size, but with the bottom surface convex so that the prepared tablet would have a concave rather than a flat surface. The urine could then be dropped onto the concave surface of the indented tablet.

*B. Procedure (Fig. 1, A, B, C)*—Using an ordinary eye dropper or a pipette measuring 20 drops to the cubic centimeter, 10 drops of urine are dropped slowly upon the surface of the tablet so as not to cause any overflow. If bilirubin is present in the urine, a fine layer of yellow bile pigment will be adsorbed upon the surface of the tablet. The remainder of the urine will filter through. One or two drops of Fouchet's reagent are then dropped directly on the yellow area. A positive test is denoted by a blue-green color, which varies in intensity with the amount of bilirubin present. The test is read in the same manner as the Harrison strip test: trace to 4 plus. However, with urine containing low concentrations of bilirubin, the color change is much sharper with the tablet test. Normal urinary pigments give neither the yellowish discoloration on the tablet surface nor a positive test with the use of Fouchet's reagent. After addition of Fouchet's reagent, the green color should be read immediately, or its intensity will decrease. When no positive test is secured after addition of Fouchet's reagent, and it is still suspected that there might be a very faint trace of bile present, the use of 15 drops instead of 10 (the tablet will usually become flooded with more than 15 to 20 drops) may bring out faint traces of bile. This can be seen as a faint yellowish discoloration and will give a very faint but perceptible bluish-green color after the addition of Fouchet's reagent. Occasionally in concentrated normal urines, as with the Watson modification of Harrison's strip test, a gray or lavender color develops after the addition of Fouchet's reagent. This is not due to bile pigment, but probably indicates dehydrogenation products of urobilinogen.<sup>13</sup>

#### DISCUSSION

This tablet test has been performed on more than 1,000 urine specimens. There have been no false positive results. In many instances it has been compared simultaneously with other urine tests, such as the foam nitric acid, iodine, methylene blue, and Watson's modification of the Harrison spot test (Table I). It is inexpensive to prepare, simple to perform, more sensitive and easier to read than any of the afore-mentioned tests. We have obtained a positive test with urines containing as little as 0.25 mg. bilirubin, although at these very low concentrations the test becomes difficult to read. We are attempting at present to correlate the degree of positivity of the test with bilirubin levels in the blood.

\*Rubber Maid Door Mat manufactured by the Wooster Rubber Company, Wooster, Ohio.

Ordinary urine pigments give no yellowish color on the tablet surface nor any bluish green color after Fouchet's reagent is added. Certain therapeutic agents, such as riboflavin, Atabrine, and Pyridium which discolor urine, may give a slight yellowish discoloration of the surface of the tablet. The first two, however, give no such discoloration with ordinary therapeutic doses. None of these drug pigments give a positive test after the addition of Fouchet's reagent. Urine from patients with hematuria will also discolor the surface of the tablet but no green color is produced after the addition of Fouchet's reagent. We have noted a purplish black pigment adsorbed onto the tablet surface from the urine of a patient with acute porphyria. This tablet when held under a Wood's ultraviolet lamp gave the red fluorescence of a porphyrin. When bile pigment is present with any of these extraneous pigments a positive test is still secured with Fouchet's reagent, although it is somewhat altered by the presence of these other pigments. The intensity of the yellowish color on the tablet surface or the green surface or the green color after the addition of Fouchet's reagent can be utilized to determine semi quantitatively the amount of bile pigment in the urine as Watson<sup>13</sup> has done with the Harrison strip test. The yellow color can be used provided the patient has not been given any Atabrine, riboflavin or Pyridium. With the therapeutic doses of Atabrine or riboflavin used routinely the urinary excretion of these drugs does not discolor the surface of the tablet to any noticeable degree. However, none of the drugs interfere with the green color obtained after the addition of Fouchet's reagent. A urine containing bile can be analyzed by a quantitative method such as the Gibson\* method or the Godfred<sup>10</sup> modification of the Hunter diazo method. Various dilutions can be made and the test run upon these different dilutions of the urine of known bilirubin pigment concentration. Either the initial yellow color or the green color after the addition of Fouchet's reagent can be used to determine the quantity of bilirubin. Color photographs or drawings can be made of these different intensities which can then serve as standards for a given batch of tablets.

The formula for plaster of Paris is  $\text{Ca SO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$ . After addition of water the dry plaster sets to a coherent solid which is composed of interlacing crystals of gypsum ( $\text{Ca SO}_4 \cdot 2 \text{H}_2\text{O}$ ).<sup>14</sup> When urine containing bile is dropped upon the tablet surface the gypsum particles apparently adsorb the pigment as does talc in the Naumann test. There may be an added chemical adsorptive effect because of the presence of calcium in the gypsum molecule. Addition of barium chloride in the preparation of the tablet definitely increases the

Gibson's method for quantitative urinary bilirubin

Barium acetate 0.5 gm. is introduced into a 15 ml. centrifuge tube and 10 ml. of urine (pH 5 to 6) are poured into it and the tube is thoroughly shaken. The tube is centrifuged and after fifteen minutes the supernatant liquid is poured off and the wall of the tube and the precipitate are washed gently with water and the tube is drained. One milliliter of Ehrlich's diazo reagent is added and the precipitate is thoroughly stirred with a glass rod. Two milliliters of water, 1 ml. of saturated ammonium sulfate solution (again stirring), 8 ml. of alcohol and 1 ml. of concentrated hydrochloric acid are then added. Let stand with occasional shaking and filter (Whatman No. 5 paper). Read in a Coleman Junior spectrophotometer in an 85 by 1 mm. cuvette, wave length 575 with a water reference. If too strong the filtrate may be diluted appropriately with a blank solution. The graph secured with the spectrophotometer on semilogarithmic paper is a straight line from 0 mg. per cent at 100 per cent transmission to 1 mg. per cent at 24 per cent transmission when 10 ml. of urine are precipitated.

bilirubin adsorption The addition of Fouchet's reagent oxidizes the bilirubin to green biliverdin This bluish-green color tends to fade and become less intense after several minutes, especially with urine containing very small amounts of bile The principle of using a plaster of Paris tablet as an adsorbing filter could be applied to other substances such as porphyrin and melanin We are at present investigating this possibility

#### SUMMARY

1 The preparation and use of an inexpensive, simple, sensitive table test for detection of urinary bilirubin are described This test is a modification of Naumann and Harrison spot tests for bilirubin

2 The test makes use of a plaster of Paris tablet, made with a solution of saturated barium chloride or tap water, upon the surface of which urine is dropped The addition of Fouchet's reagent to the bilirubin pigment which is adsorbed on the tablet surface gives a clear-cut easily readable, bluish-green color which is specific for bile The test can be used for mass and serial testing and can be made semiquantitative for the determination of urinary bilirubin

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# TECHNIQUES TO OVERCOME THE LACK OF RARE RHESUS ANTISERA AND CELLS\*

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COMPARATIVELY few laboratories can rely on obtaining supplies of the rarer Rhesus antisera such as pure anti C (*anti rh'*) and pure anti E (*anti rh''*), nor are donors of the rarer blood cells Cde/ede or Cde/Cde (*rh'*) and eDE/ede or eDE/eDE (*rh''*) readily available. Most laboratories undertaking Rh typing will however have supplies of the mixed antisera C + D (*anti Rh<sub>0</sub>'*) and D + E (*anti Rh<sub>0</sub>''*) as well as complete and incomplete pure anti D (*anti Rh<sub>0</sub>*).

The preparation of anti C (*anti rh'*) and anti E (*anti rh''*) from mixed sera is often unsatisfactory. Dilution removes the anti C and anti E which are almost invariably of lower titer than the anti D (*anti Rh<sub>0</sub>*), and when an absorption technique is employed the titer of the anti C and anti E already low as a rule is still further reduced.

The lack of the rare cells Cde/ede or Cde/Cde (*rh'*) and eDE/ede or eDE/eDE (*rh''*) prevents the testing of sera for anti C or anti E in the presence of anti D, the sera having to be stored until these cells are available while the lack of pure anti C (*anti rh'*) or anti E (*anti rh''*) causes difficulties in genotyping.

Techniques are described below which overcome these difficulties. They have been used in this laboratory with satisfactory results having been controlled by the use of pure anti C (*anti rh'*) and anti E (*anti rh''*) and by fresh cells of the types Cde/ede and eDE/ede (*rh'* and *rh''*).

The principle on which they are based is the exclusion of the D antigen (*R<sup>0</sup>*) in Cde/ede (*R<sup>1</sup>*) or eDE/ede (*R<sup>2</sup>r*) cells and the D (*R<sup>0</sup>*) antibody in mixed anti C + D (*anti Rh<sub>0</sub>'*) or anti D + E (*anti Rh<sub>0</sub>''*) from the field of action by means of a strong incomplete anti D (*anti Rh<sub>0</sub>*) serum using a modification of the blocking technique originally described by Wiener.<sup>1</sup>

DETECTION OF THE ANTIBODIES C AND E (*anti rh* AND *anti rh''*) IN THE PRESENCE OF ANTI D (*anti Rh<sub>0</sub>*) BY MEANS OF CELLS OF THE COMMON GENOTYPES CDE/CDE AND CDE/CDf (*R<sup>1</sup>* AND *R<sup>2</sup>*) —

"Prepare cells of the comparatively rare genotypes Cde/ede (*rh'*) and eDE/ede (*rh''*) from cells of the common genotypes CDE/ede (*R<sup>1</sup>*) and eDE/ede (*R<sup>2</sup>r*) as follows

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At the present time two systems of nomenclature of the anti Rh typing sera and cells are in wide use. One of the systems is based on the series of allelic genes proposed by Wiener the other on a series of closely linked pairs of genes proposed by Race and Fisher of England. A comparison of the individual anti sera has been made recently by the advisory board of the National Institute of Health. In this paper the Race-Fisher nomenclature has been employed but for the convenience of the reader Wiener's nomenclature is also given in parentheses and italics.

1 Subject a saline suspension of the cells CDe/cde ( $R^1$ ) or cDE/cde ( $R^2r$ ) to the action of a strong incomplete (blocking) pure anti-D (*anti-Rh<sub>0</sub>*) serum for one and one-half to two hours at 37° C

2 Examine for absence of agglutination and confirm "coating" by means of Coombs reagent (antihuman globulin serum)

These "prepared" cells can now be used to detect the presence or absence of anti-C (*anti-rh'*) or anti-E (*anti-rh''*) in a serum known to contain anti D (*anti-Rh<sub>0</sub>*)

3 Divide the cell suspension into three portions—one test and two controls

Test one with the serum under examination, incubating for one and one-half to two hours at 37° C

Agglutination indicates the presence and no agglutination the absence of anti-C (*anti-rh'*) or anti-E (*anti-rh''*)

Test two with a saline agglutinating anti-D (*anti-Rh<sub>0</sub>*) serum having a titer comparable with that of the anti-D in the mixed serum, incubating for one and one-half to two hours at 37° C

Incubate the third at the same time as one and two

No agglutination should occur in two and three

DETECTION OF THE ANTIGENS C ( $r'$ ) AND E ( $r''$ ) IN THE PRESENCE OF D ( $R^0$ ) BY MEANS OF MIXED ANTI-C + D (*Anti-Rh<sub>0</sub>'*) AND ANTI-D + E (*Anti Rh<sub>0</sub>''*) SERA —

1 Subject a saline suspension of the cells under examination to the action of an incomplete (blocking) pure anti-D (*anti-Rh<sub>0</sub>*) serum for one and one half to two hours at 37° C

2 Examine for absence of agglutination and confirm "coating" by means of Coombs reagent

These "prepared" cells are now magglutinable by anti-D (*anti-Rh<sub>0</sub>*), and mixed anti-C + D (*anti-Rh<sub>0</sub>'*) or anti-D + E (*anti-Rh<sub>0</sub>''*) serum can be used to detect in them the antigen C ( $r'$ ) or E ( $r''$ )

3 Divide the cell suspension into three portions—one test and two controls

Test one with the mixed anti-C + D (*anti-Rh<sub>0</sub>'*) or anti-D + E (*anti Rh<sub>0</sub>''*) serum, incubating for one and one-half to two hours at 37° C

Agglutination indicates the presence and no agglutination the absence of the antigens C ( $r'$ ) or E ( $r''$ )

Test two with a saline agglutinating anti-D (*anti-Rh<sub>0</sub>*) serum having a titer comparable with that of the anti-D in the mixed serum, incubating one and one-half to two hours at 37° C

Incubate the third at the same time as one and two

No agglutination should occur in two and three

NOTE—Where cells of the genotype Cde/cde ( $r^1$ ) or cDE/cde ( $r^{11}$ ) are available, attention should be drawn to the fact that a proportion of them are CD<sup>e</sup>e/cde or cD<sup>e</sup>E/cde (Stratton)<sup>2</sup> A preliminary investigation in this laboratory shows that about 10 per cent of so-called Cde/cde ( $r^1$ ) cells are CD<sup>e</sup>e/cde. The technique outlined herein for the "preparation" of cells will

prevent errors through using a  $CD^{u}e/cde$  cells thought to be  $Cde/cde$  ( $r'$ ) or a  $cD^{u}E/cde$  cell thought to be  $cdE/cde$  ( $r''$ )

Wiener's nomenclature<sup>3, 4, 5</sup> is given in parentheses and italics for the convenience of those laboratories which still adhere to it, but difficulty is found in expressing the various allelomorphs of D, C, and c which are now known to exist, e.g., in this paper no equivalent in Wiener's nomenclature is suggested for the cells  $CD^{u}e$  and  $cD^{u}E$  thus may be left to the ingenuity of the reader

Thanks are due the Regional Transfusion Officer Lt Col R H Malone, for permission to publish this note and for his suggestions also to Dr R R Race of the M R C Blood Group Research Unit Lister Institute for his continued advice and encouragement, and especially to Mr F Gould of our Transport Department for his willing donations of  $cdE/cdE$  ( $r''r''$ ) cells wherever required

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# A GALVANOTACTIC PROCEDURE FOR THE CONCENTRATION OF BALANTIDIUM COLI IN FECES

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## INTRODUCTION

THE diagnosis of balantidiasis is seldom established by physicians because the disease is little known. Another important reason is that *Balantidium coli* are seldom found in fresh stool specimens. The common helminthological and parasitological methods of stool examination utilize only a small quantity of fresh material and often kill or injure the balantidia. By examining stool specimens diluted with physiologic saline, Stschensnowitsch in Azerbaijan<sup>1</sup> was able to increase materially the percentage of positive results. He found *B. coli* in 56 (51 per cent) of 1,100 cases. Before this study, only four cases of balantidiasis were discovered in the whole of Azerbaijan.

The employment of a procedure which would increase the recovery of parasites from stools would be of value in the diagnosis of balantidiasis. The idea of concentrating the parasites by employing the phenomenon of galvanotaxis used in the study of free living Infusoria and of parasitic Infusoria of frogs<sup>2, 3</sup> suggested itself. The apparatus, which utilizes a galvanic current and is described below, has been successfully used for concentrating Infusoria from the rectum of the frog and *b. coli* from the feces of swine.

## MATERIALS AND METHODS

**Apparatus**—As a source of current, a Kenotron rectifier with a potentiometer was used, but any standard rectifier is suitable. The apparatus produced a direct current of 132 volts. Preliminary investigations were set up in a chambered slide. These chambers were constructed with glass strips 2 mm thick and 5 mm wide and sealed with Canada balsam in such a way that three chambers of not quite equal size resulted (Fig. 1). These chambers were connected by means of channels 5 by 5 by 2 mm in size. The channels were initially filled with 2.5 per cent agar (pH 7.5) in Ringer's solution. The central chamber was filled with a balantidia containing fecal suspension in Ringer's solution and covered with a cover glass. The side chambers were half filled with Ringer's solution in which were immersed pipette like agar bridges connecting them with Petri dishes containing Ringer's solution. The Petri dishes were connected by means of agar bridges with other dishes containing saturated copper sulfate solution in which were immersed copper electrodes. The resistance between the electrodes was approximately 30,000 to 35,000 ohms. The diffusion of copper ions into the chamber may be practically eliminated by changing the Ringer's solution in the dishes at one half to one hour intervals.

Warming the chamber to 37 to 42° C facilitated the collection of balantidia at the cathode. Therefore, in the construction of a practical apparatus, a means of warming the chamber as well as increasing its volume had to be considered.

As concentration vessels, stool sample tubes 70 mm in length and 22 mm in diameter were chosen. These are immersed in a water bath in such a manner that they project a distance of about 1 cm over the cover plate, a rubber ring being placed about the tube



at this level. The anodic agar bridge was dipped a few millimeters into the suspension. The cathodic bridge is inserted into the upper dilated end of a special concentration tube, suspended in the stool sample tube by a glass hook (Figs 2 and 3). The lower end of the concentration tube is closed by a porcelain filter and the tube filled with Ringer's solution.

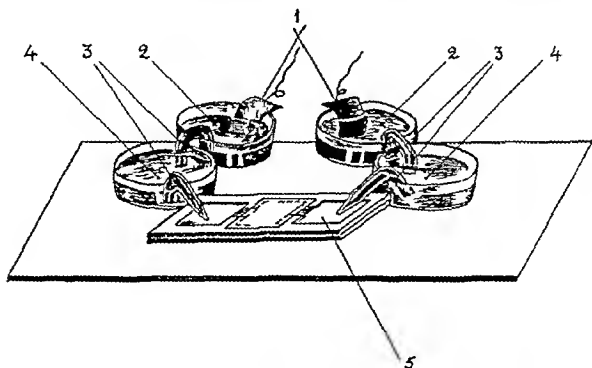


Fig. 1—Chamber apparatus for concentrating Infusoria from rectum of frog

- 1 Copper electrodes
- 2  $\text{CuSO}_4$  solution
- 3 Agar bridges
- 4 Ringer's solution
- 5 Galvanotactic chamber

A short length of rubber tubing is placed about the end of the tube which does not reach the bottom of the stool sample tube. The balantidia collect inside the lumen of the rubber tube below the porcelain filter (Figs 2 and 3).

In filling the concentration tube and rubber tube with Ringer's solution, no air bubbles must be permitted to enter since these greatly increase electrical resistance. A cellophane cap may be used in place of the porcelain filter. If cellophane is used, one must be sure that no portion protrudes beyond the rubber tubing since balantidia would also collect there. Instead of the concentration tube described one may use a long cathodic agar bridge which is filled with agar except for about 5 mm. at the lower end, this space being filled with Ringer's solution. Such an agar bridge may be used only once since the balantidia migrate into the capillary space between the wall of the tube and the agar. The concentration tube equipped with the porcelain filter need only be immersed in boiling water before reusing. The concentration process was carried on for a period of ten minutes at 39° C. The resistance when the porcelain filter was used was about 35,000 to 40,000 ohms, with cellophane 20,000 to 25,000 ohms. The current strength was 0.3 to 6.0 milliamperes.

Then the concentration tube was removed from the stool sample tube and the contents of the concentration space (below the porcelain plate) were pipetted out and examined microscopically.

**Staining Specimens.**—For counting the *B. coli* a small drop of saturated potassium iodide iodine solution was added. For nuclear staining a mixture of equal parts of a saturated aqueous solution of methyl green and 40 per cent formaldehyde (formalin) was employed.

In our material (cecal contents of swine) balantidia of the suis type with long sausage shaped nuclei predominated. Balantidia with round or bean shaped nuclei were seen only in about 15 per cent of swine. They formed less than 1 per cent of the whole balantidial population. The stool specimens were examined between two and six hours after the swine were killed.

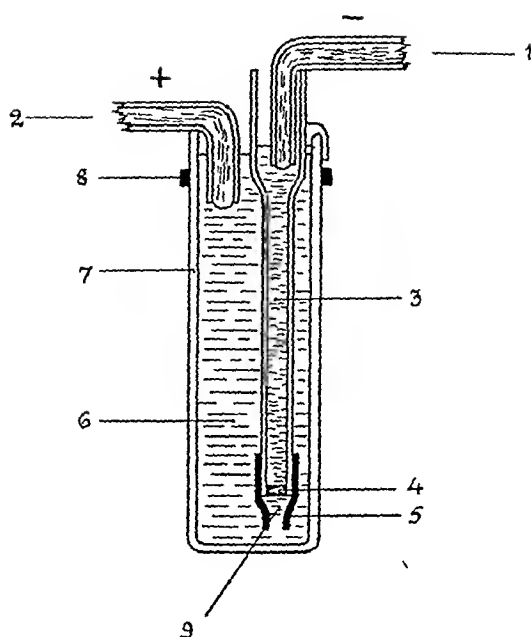


Fig. 2—Concentration tube for collecting *B. coli*

- 1 Cathodic agar bridge
- 2 Anodic agar bridge
- 3 Concentration tube
- 4 Porcelain filter
- 5 Rubber tubing
- 6 Suspension of feces in Ringer's solution
- 7 Test tube
- 8 Rubber ring
- 9 Concentration space (trap)

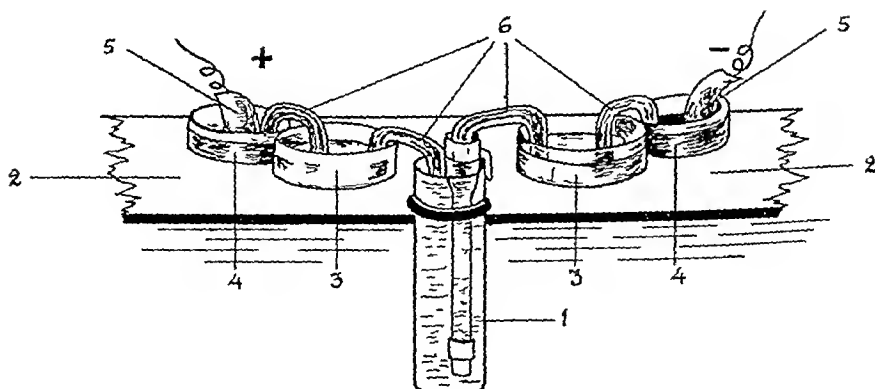


Fig. 3—Concentration tube with electrical connection for collection of *B. coli* from swine feces

- 1 Test tube
- 2 Lid of the water bath
- 3 Petri dishes with Ringer's solution
- 4 Petri dishes with copper sulfate solution
- 5 Copper electrodes
- 6 Agar bridges

The preliminary observations employing the apparatus shown in Fig 1 were done using Infusoria from the frog rectum and swine balantidia *Balantidium coloon* (frog) and also the swine balantidia collected at the cathode at room temperature, with a current strength of 0.3 to 1.0 Ma for three to five minutes, and remained there as long as the current was flowing. Under the same conditions *Opalina ranarum* and *Opalina dimidiata* collected at the anode where they soon were killed. Apparently it is possible to separate single species of ciliophoria from a mixed population in establishing the necessary current strength for the anadromous movement of different species.

The stool specimens taken from swine ceca were first investigated without concentrating and four cover glass preparations of sediment of a fecal suspension in a Ringer's solution (1:3) were examined at low magnification. Then the galvanotactic chamber (Fig 1) was filled with the same sediment and the inside surface of the agar cathode was examined at low magnification. Of twenty stool specimens investigated in this manner balantidia were found without concentration in eight specimens while with concentration they were found in twelve specimens. The percentage of positive results was increased therefore only from 45 to 60 per cent which was to be expected since the volume of the chamber (0.45 ml) is hardly greater (Fig 1) than the sediment observed under the four cover glasses.

*Optimal Position of the Electrodes*—Fifteen milliliters of a 1 to 3 dilution of stool suspension were placed in each of two sample tubes (Fig 2) and concentrated for ten minutes at 39° C using a current of 3 milliamperes. With one of the samples the long cathodic concentration tube described was used. With the second sample a long anodic agar bridge was employed with a short cathodic concentration tube which extended only 1 cm beneath the surface. In Table I are shown the numbers of balantidia 'captured' at different electrode positions.

TABLE I. POSITION OF ELECTRODES IN RELATION TO RECOVERY OF *B. COLI*

EXPERIMENT	WITHOUT CONCENTRATION*	WITH CONCENTRATION	
		SHORT CATHODE	LONG CATHODE
1	0.25	0	0
2	3.75	92	174
3	1.75	23	98
4	0.75	1	45
5	0	0	24
6	0	2	17

The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

The best results were obtained when the cathode was near the bottom of the stool sample tube—a fact probably dependent on the positive geotaxis of balantidia.

*Optimal Temperature*—Each stool suspension was divided into six equal portions and concentrated at temperatures ranging from 25° to 50° C with a current strength of 3 Ma for ten minutes. The results are shown in Table II.

TABLE II TEMPERATURE OF FECES SUSPENSION IN RELATION TO RECOVERY OF B COLI

EXPERIMENT	WITHOUT CONCENTRATION*	WITH CONCENTRATION					
		25° c	30° c	35° c	40° c	45° c	50° c
1	3.5	2	14	73	126	83	0
2	2.5	2	9	8	100	15	0
3	0	0	2	4	4	0	0
4	0.75	8	31	115	135	14	0
5	1.0	0	5	17	52	43	0

\*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

The optimal temperature is 40° C, as Table II shows. At 25°, the balantidia are not concentrated by this apparatus and between 45° to 50° the Infusoria died. A temperature of 39° C was thereafter chosen for our experiments, corresponding to the rectal temperature of swine.

*Duration of Concentration*—In order to determine the most practical time interval for concentrating the balantidia, experiments were done lasting eighty minutes at a temperature of 39° C, with a current strength of 1 mill ampere. Every ten minutes the concentration tube was removed and the balantidia in the trap were counted. The results are given in Table III.

TABLE III TIME NECESSARY FOR MAXIMUM CONCENTRATION OF B COLI

EXPERIMENT	WITHOUT CONCENTRATION*	NUMBER OF BALANTIDIA RECOVERED AT							
		10 MINUTE INTERVALS WITH CONCENTRATION							
		1	2	3	4	5	6	7	8
1	0	18	15	4	1	—	—	—	—
2	4.0	1,732	168	85	53	38	27	51	22
3	3.0	938	105	54	29	11	6	5	6
4	0.5	35	14	11	5	2	—	—	—
5	0	3	—	—	—	—	—	—	—

\*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

As is shown in Table III, about 50 to 80 per cent of balantidia were obtained in the first ten minutes. The number of balantidia diminished rapidly in later samples, although at the end of eighty minutes some still were present in two samples. Therefore, if no balantidia are obtained in the first ten minutes, further stool suspension should be investigated. Similar results were obtained when a current of 3 Ma was used.

*Optimal Current Strength and Its Relationship to the Age of the Stool Sample*—In determining the optimal amperage, four tubes were filled with stool suspension and currents of varying intensity (0.3, 1.0, 3.0, and 6.0 Ma) were passed for ten minutes at 39° C. The optimal current strength for concentrating balantidia from fresh stool samples was mostly between 1 and 3 millamperes. In older stool samples, optimal current strength varies so that we were obliged to repeat our experiments until balantidia could no longer be found. They were found to persist in stool samples a maximum of ten days at room temperature. This is in contradiction to usually accepted ideas. Table IV shows the results.

TABLE IV OPTIMAL CURRENT STRENGTH AND AGE OF FECES SAMPLE IN RELATION TO RECOVERY OF BALANTIDIA

POOM TEMPERATURE 22 26 c											
AGE (DAY)	STOOL SAMPLE 85			STOOL SAMPLE 87			STOOL SAMPLE 88		STOOL SAMPLE 89		
	1	2	3	1	2	3	1	2	1	2	3
Without concentration*	25	0	0	275	0	0	0	0	125	0	0
03 Ma	27	5	0	252	0	0	1	0	13	0	0
10 Ma.	54	18	0	305	4	0	5	0	48	5	0
30 Ma	57	35	0	372	11	0	1	0	69	5	0
60 M <sub>1</sub>	30	11	0	299	10	0	1	0	174	14	0

AGE (DAYS)	STOOL SAMPLE 90		STOOL SAMPLE 91		STOOL SAMPLE 92		STOOL SAMPLE 93		STOOL SAMPLE 94	
	1	2	1	2	1	2	1	2	1	2
Without concentration	0	0	0	0	0	0	25	0	0	0
03 Ma	0	0	0	0	0	0	15	0	0	0
10 Ma	0	0	0	0	0	0	150	0	2	0
30 Ma	1	0	1	0	0	0	97	0	1	0
60 M <sub>1</sub>	0	0	0	0	2	0	62	0	2	0

AGE (DAYS)	STOOL SAMPLE 86									
	1	2	3	4	5	6	7	8	9	10
Without concentration*	405	725	40	10	025	10	175	10	0	0
03 M <sub>1</sub>	2839	42	97	6	3	0	2	1	2	0
10 M <sub>1</sub>	3639	389	86	50	24	6	1	6	0	0
30 Ma.	3803	490	84	125	107	23	41	11	2	0
60 Ma	2479	547	322	280	53	51	52	2	0	0

The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

It is apparent that balantidia remain viable up to ten days. In Fig 4 the percentage of positive findings after concentration are plotted against the age of the stool sample. All twenty five samples were positive on the first day. The disappearance of balantidia from the stool samples does not depend wholly on a change in pH. They may disappear even in neutral samples (pH 7.0 to 7.2). If numerous starch granules are present they may sometimes be found in acid stools (up to pH 5.6). When the concentrating procedure was used, balantidia could frequently be demonstrated one day later than without concentration.

The optimal current strengths tend to increase with the age of the stool sample. After several days, this amounts to 3 to 6 milliamperes. Such amperages are, however, also optimal for some fresh stool samples containing only few balantidia. The effect of pH on the optimal current strength must be further investigated.

We have examined one hundred stool samples from swine feces at a temperature of 39° C, current strength 3 Ma for ten minutes. Without concentration 56 per cent were positive with concentration, 92 per cent were positive. The results of twenty of these experiments are shown in Table V.

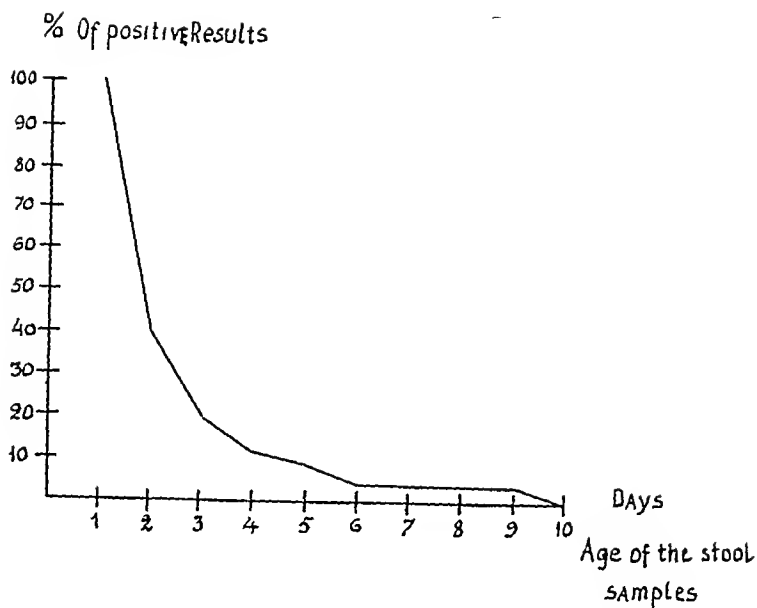


Fig 4—The recovery of *B. coli* in relation to age of stool samples  
All twenty five samples were positive on the first day

TABLE V NUMBER OF *B. COLI* OBSERVED WITH AND WITHOUT CONCENTRATION IN TWENTY DIFFERENT SAMPLES OF FECES

NUMBER OF BALANTIDIA			NUMBER OF BALANTIDIA		
SAMPLE	WITHOUT CONCENTRATION*	WITH CONCENTRATION	SAMPLE	WITHOUT CONCENTRATION*	WITH CONCENTRATION
1	4.25	186	11	4.0	1,732
2	99.75	3,927	12	3.75	57
3	6.75	354	13	0.5	9
4	41.0	4,230	14	0	3
5	9.0	299	15	0	0
6	1.25	52	16	5.25	436
7	11.5	4,430	17	0	10
8	26.25	3,312	18	2.5	100
9	2.5	84	19	0	4
10	0	18	20	0.75	112

\*The numbers in this column give the mean of four cover slip preparations. The material was taken from the sediment in the bottom of the stool sample tube before beginning concentration.

It is seen that the number of balantidia after concentration is not directly proportional to the number obtained without concentration. For example, in Sample 13 the increase was eighteen fold, while in Sample 11, it was four hundred twenty fold. It is possible that these results might be improved by taking into consideration other factors, such as pH.

On examining fifty stool specimens from swine from another region, the positive results were increased from 34 per cent to 52 per cent by using the concentration procedure.

#### SUMMARY AND CONCLUSIONS

1. A galvanotactic method for concentrating *B. coli* from swine feces has been described.

2 By using this concentration procedure, the percentage of positive results has been substantially increased from 56 per cent to 92 per cent in one hundred samples and from 34 per cent to 52 per cent in fifty samples from swine from a different locality

3 It is suggested that this galvanotactic method also may be employed for concentrating *B. coli* and other protozoan parasites in the feces of man

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This observation applies similarly to the globulin subfractions and is confirmed by the electrophoretic diagram before and after performance of the cadmium reaction (Fig 1). The fairly regular diminution of all peaks is easily recognizable.

In the myeloma Serum 3 (Table I) where three-fourths of the serum proteins consist of gamma globulin, the cadmium precipitate has a corresponding imbalance (Fig 2). Probably this would have been even more pronounced if it had not been necessary for technical reasons to keep the total protein content for the three electrophoreses constant. The relative increase of albumin content is thus also explained, however, calculation shows that the albumin in Serum 3 participated only 6.1 per cent in the diminution. In Sera 2 and 1, where the albumin contents were twice and three times that in Serum 3, the proportional figures of the diminution were 3 and 6.4 times as much albumin. Thus in the case of gamma myeloma (Serum 3) less albumin is precipitated than in the other sera. Moreover, in Serum 2, where the albumin and gamma globulin contents were nearly the same, the decrease in albumin amounted to 18.6 per cent, yet that in gamma globulin was 33.0 per cent. Hence it follows that the cadmium reagent precipitates more gamma globulin than albumin.

Alpha and beta globulin are precipitated to a smaller extent, although further investigations are necessary to decide which of these subfractions is predominantly included.

Summarizing, electrophoretic analysis of three pathologic sera with strongly positive cadmium reaction leads us to conclude that the cadmium reagent precipitates gamma globulin primarily, but that alpha and beta globulin and albumin are also included in the precipitate. In this respect the composition of the initial serum, which is relatively dependent on disease, is of decisive importance. In general, the higher the content of one subfraction as compared with the others, the more strongly is it affected by the reagent. Since it is first of all a question of an anion action (the  $\text{SO}_4$  ion having a strongly dehydrating action according to its position in the lyotropic group) all protein fractions are affected. Thus the cadmium reagent differs greatly from the thymol and cephalin-cholesterol reagent.

According to Recant, Chaigaff, and Hanger<sup>7</sup> (1945), increased hpo proteins lead to a positive thymol test, though they influence the cephalin test but little, they state that the latter reacts considerably to an increase of gamma globulin, whereas thymol shows little or no reaction. Cohen and Thompson<sup>16</sup> (1947) obtained similar results and found a strong thymol turbidity each time the lipid-carrying beta globulins were markedly increased. This correlation was supported also by electrophoresis after centrifugation of the precipitate, which forms after standing overnight. Since then, Kunkel and Hoagland<sup>8</sup> (1947) have shown by electrophoresis of three thymol-positive sera that this reagent combines chiefly with beta globulin, and to a lesser degree with gamma globulin. In this case also the relative quantity precipitated is in close relation to the protein composition of the initial serum. The complex composition of the precipitate shows that probably one grasp



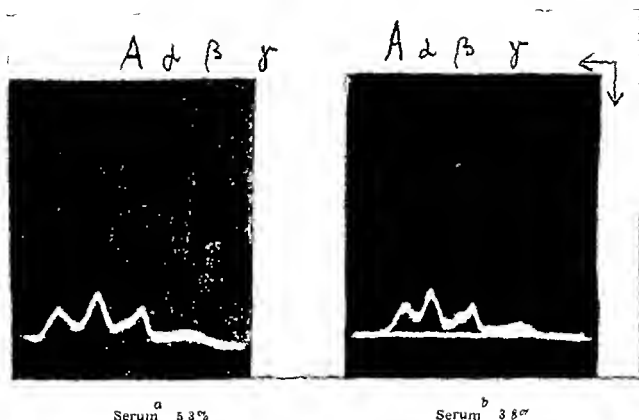


Fig 1—A 68 year old woman pulmonary tuberculosis amyloidosis of the kidneys  
<sup>a</sup> Native serum  
<sup>b</sup> Serum after precipitating with  $\text{CdSO}_4$  equal decrease of every fraction the total diminution of the serum proteins is 30% per cent or 1.52 Gm. per cent (see Table II)  
 Electrophoresis descending boundaries  
 Temp 3 Cel pH = 7.9  $\mu = 0.1$  Buffer Michaelis barbiturate

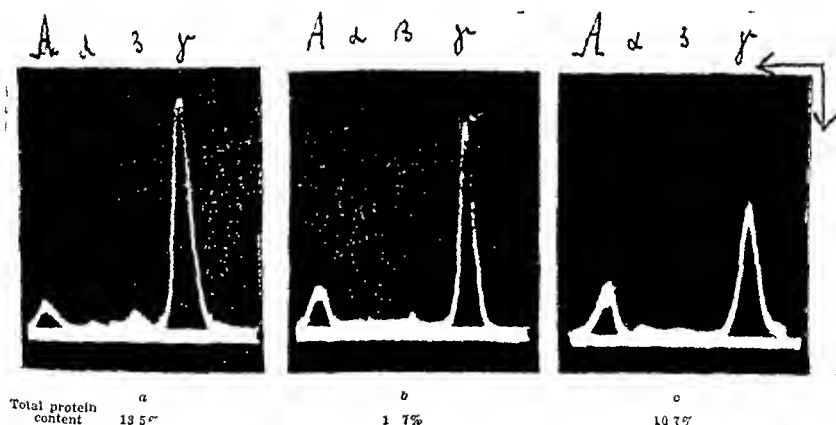


Fig —A 63 year old woman multiple myeloma (see Table II)  
 Effect of the  $\text{CdSO}_4$  reagent  
<sup>a</sup> Native serum  
<sup>b</sup> Serum after  $\text{CdSO}_4$  precipitating decrease of the γ globulin fraction  
<sup>c</sup> Serum after repeat  $\text{CdSO}_4$  precipitating further decrease of the γ globulin fraction  
 Electrophoresis descending boundaries  
 pH = 7.9  $\mu = 0.1$  temp 3 Cel Buffer Michaelis barbiturate

of these reactions between reagent and protein subgroups is not yet complete. Verification of the precipitation optima by MacLagan and Bunn<sup>9</sup> (1947) on electrophoretically-isolated protein fractions suggested, however, a greater susceptibility of the cephalin-cholesterol reagent to gamma globulin. This was found to be increased about ten times with gamma globulin taken from a hepatitis serum. This is at variance with the findings of Kabat, Hanger, Moore, and Landow<sup>10</sup> (1943) who observed a similar precipitation with the cephalin test, and also with the colloidal gold test, even when the gamma globulin had been isolated from different patients. MacLagan<sup>13</sup> found no reaction between isolated alpha and beta globulins and the thymol reagent. He concludes that the latter reacts with gamma globulin. Further investigations are necessary to clear up this question, including the intense yet varying inhibitive action of albumin (Kunkel and Hoagland<sup>8</sup>) and the alterations in precipitative ability of the individual reagents in disease connected with their varying response in the course of the illness. It also has been proved that during convalescence the individual tests remain positive for varying lengths of time. (See Gray and Barron,<sup>11</sup> Martin,<sup>12</sup> Maizels<sup>13</sup>)

For clinical needs simultaneous application of several tests to the same case is to be recommended at present. As we have already shown (Wuhrmann, Wunderly, and Bubb<sup>14</sup>) the average response to the cephalin and thymol reaction agrees to a large extent in extensive series of tests on three different disease groups (hepatic disorders, infectious diseases without participation of the liver, pulmonary tuberculosis). This is true also when the cadmium test is included in such a comparison (Table II). On the other hand, parallel tests performed simultaneously often exhibit divergences, sometimes one, sometimes another test appearing more sensitive. These individual deviations are only equalized, as Table II shows, to a very considerable extent, when results are taken as a whole.

TABLE II

	HEPATIC DISORDERS	INFECTIOUS DISEASES (WITHOUT PARTICI- PATION OF THE LIVER)	PULMONARY TUBERCULOSIS
	NUMBER OF CASES EXAMINED		
	180	135	106
Positive result			
Cephalin test	126	84	55
Thymol test	131	82	60
Cadmium test	123	93	68

We have already drawn especial attention to the significant fact that under pathologic conditions a globulin increase (of one or more subfractions) together with a simultaneous albumin decrease is always observed in every case, and that among many thousands of investigations we never observed an albumin increase. The observation of this one-sidedly inverse regulation mechanism is thus of especial importance to the result of serum lability tests, since the positive result of these tests is dependent on an increase of one or

several globulin fractions, the albumin always appearing diminished at the same time. Precipitation of the albumin by the cadmium reagent never occurs in normal plasma under the test conditions described, in the case of disease occasioned alterations in the serum, where precipitation of the albumin may occur by means of the cadmium reagent the albumin is always quantitatively diminished. In exactly the same way, the quantitative decrease of the albumin always also appears to be connected with qualitative alterations as is observed in the increase of the globulin fractions connected therewith. It remains to be seen whether in disease the two fractions of albumin as they were shown by Luetscher<sup>17</sup> (1939) at pH 4, have different ratios from normal serum. An increase in one of them might easily lead to a different reactivity with the reagents of the lability tests. As a rule albumin does not split up into several underfractions at the pH range in which electrophoresis is usually carried out and we are therefore, not well informed on the changes in the albumin fraction. According to our experience also the lipid content of the serum (and thus the beta globulin complex) plays a particular part in the thymol turbidity test (Kunkel and Horgland<sup>8</sup> Hange<sup>1</sup>).

The view that an actual specificity exists in all these tests in respect to hepatic diseases or parenchymatous damage to the liver finally has been abandoned (Wuhrmann Wunderly and Bubb<sup>14</sup>). For the cadmium reaction we have emphasized from the beginning that clinical evaluation must make a strict distinction between afebrile disease (positive result especially in *hepatic* and *renal* diseases) and fever states in which the positive result is generally speaking an expression of the reaction of the reticuloendothelial system. Table II shows that this observation also applies to the cephalin cholesterol and thymol tests.

#### SUMMARY

1 The cadmium reaction a simple test utilizing a stable reagent and five minute reading is described. According to electrophoretic tests it is not dependent on one but on several protein fractions and especially on their relationship with one another. The positive cadmium reaction is due above all to an increase of the gamma globulin but an increased content of the alpha and to a lesser extent of the beta globulins contributes. Precipitation of the albumin is likewise observed in such sera.

2 The greater the increase in content of an individual globulin fraction the more will be precipitated by the cadmium reagent. The albumin fraction precipitates only if the globulin fractions exhibit morbid displacement.

3 Attention is drawn to the clinical importance of the cadmium reaction and its delimitation in respect to the cephalin cholesterol and thymol reactions.

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# AN IMPROVED DEVICE FOR OBTAINING PLASMA ANAEROBICALLY

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THE method of centrifuging blood in the syringe in which it is drawn in order to obtain plasma anaerobically\* has proved useful in research and clinical laboratories. When the syringe is centrifuged the barrel is prevented from being forced over the plunger by means of a metal or plastic spacer held

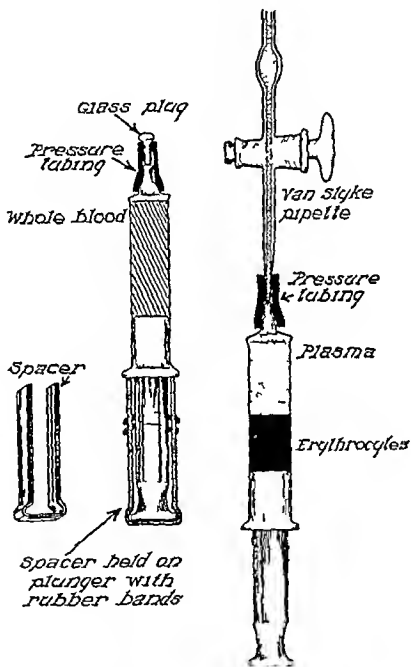


Fig 1—The use of the syringe spacer to obtain plasma anaerobically

on the plunger by rubber bands. However, construction of the spacers as originally described requires the services of a moderately skilled mechanic. The improved spacer described here can be made by anyone.

From the Department of Physiology, University of Utah College of Medicine  
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## DESCRIPTION

The spacer is made of a 1 cm wide strip of 20 gauge aluminum or other metal bent into the shape shown in Fig 1. The strip of metal can be shaped by bending it around two nails driven in a board. It is advisable to make spacers of graded sizes. The most useful sizes are those which when placed over a 5 ml syringe will permit it to hold 5, 4, or 3 ml of blood. Spacers for 2 ml syringes are particularly useful when blood is being drawn from small animals.

The entire plunger of the syringe is greased with a light-bodied stopcock lubricant. The plunger is pushed into the barrel and turned through several revolutions. A needle is placed on the syringe, and a few drops of heparin solution are drawn into it. If more than a small bubble of air remains in the syringe it is expelled. Blood is drawn into the syringe, and some is expelled for measurement of pH. The needle is removed, and a length of pressure tubing about 15 cm long is placed on the tip of the syringe. With the tip held upward the plunger is forced in to fill the lumen of the tube with blood. A glass plug, fire polished on one end and flattened into a knob on the other, is inserted into the lumen of the tubing.

The appropriate spacer is placed around the plunger and tightly wrapped with rubber bands. The plug is removed, and the plunger is pushed in until the spacer comes to rest against the barrel. While the spacer is held in this position the plug is replaced. The assembly is then centrifuged with the plunger down. When centrifugation is finished the syringe is held with tip up and the spacer is removed. The plug is withdrawn and plasma is forced into a blood gas pipette whose tip is inserted into the lumen of the tubing.

# ON THE DETERMINATION OF PROTEIN IN SERUM AND IN FRACTIONS OBTAINED FROM SERUM WITH A BIURET REAGENT PREPARED WITH SODIUM HYDROXIDE

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IN 1942 Kingsley proposed the use of a biuret reagent containing 11.7 and 17.3 per cent sodium hydroxide for the determination of total protein and albumin, respectively in serum.<sup>1</sup> Gornall and co workers have recently reported that the reagent prepared in this way may lead to serious error and they suggest the use of tartrate as a stabilizing agent.<sup>2</sup> Other workers have applied the biuret reaction in various ways.<sup>3, 4</sup> For the past two years we have employed the reagent of Kingsley with 11.7 per cent sodium hydroxide for the determination of total protein in serum and of the protein remaining in solution after the precipitation of globulin fractions with either sodium sulfate or methyl alcohol.<sup>5, 6</sup> Our experience with this reagent is quite satisfactory and we endeavor to show in this paper that it permits a convenient method for chemical laboratories.

## EXPERIMENTAL

The method of Kingsley was modified slightly in order to use the same reagent for serum and for filtrates obtained from serum. The reagent was prepared by adding 1200 ml of 1 per cent copper sulfate to 6000 ml of a solution containing 968.4 Gm of sodium hydroxide, using sodium hydroxide pellets of ACS grade from only one source\* and copper sulfate ACS fine crystals, from only one batch. In contrast to the experience of Gornall and co workers we have found the reagent prepared in this way to be stable in a stock bottle for several months and to be uniform on repeated preparation. Standard curves obtained from serial dilutions of serum or filtrates of serum of known protein content, determined by the Kjeldahl method and correction for nonprotein nitrogen have been identical. Although we have not succeeded in finding a convenient substance which gives the biuret reaction to substitute for serum in the standardization Hiller, Grief and Beckman have reported that chromium ammonium sulfate has essentially the same absorption curve as that of the biuret color.<sup>7</sup> Apparently this is not true of the color obtained with the reagent containing 11.7 per cent sodium hydroxide. Fig 1 compares the curves obtained with an Evelyn colorimeter of chromium ammonium sulfate, cobaltous sulfate, and potassium permanganate. Although neither the chromium complex nor cobalt sulfate is similar to our biuret color, potassium permanganate is quite similar and may be used for standardization. Fig 2 shows that the color of permanganate obeys Beer's law over a considerable range at 540 millimicrons.

The determination was made as follows. For total protein 0.15 ml of serum was added to 10 ml of the reagent in a colorimeter tube. About 2 ml of ether were added, and the mixture was thoroughly shaken and allowed to stand at room temperature for fifteen minutes. The color was then measured in an Evelyn colorimeter at 540 m $\mu$  using a blank solution containing 10 ml of reagent and 0.15 ml of 0.9 per cent sodium chloride. For albumin in the methyl alcohol filtrates obtained by the method of Pillemor and Hutchinson,<sup>8</sup> 1 ml of solution was added to 10 ml of reagent and the color was measured after fifteen minutes, using a blank solution containing all of the reagents. For filtrates

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obtained from serum with various concentrations of sodium sulfate, 3 ml were added to 10 ml of the biuret reagent and the color also was measured after fifteen minutes, using a blank solution containing all of the reagents

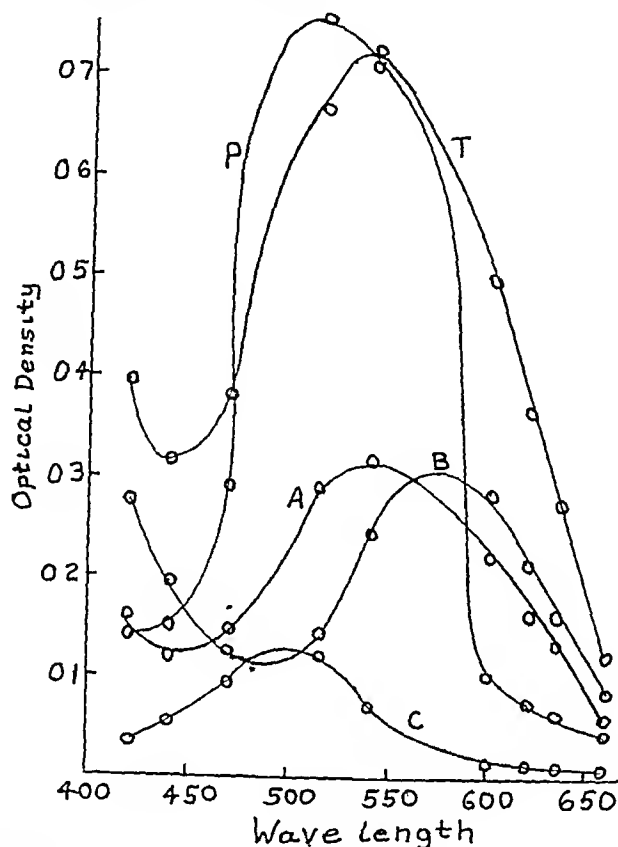


Fig 1—Absorption curves  
*T* Serum *A* filtrate of serum in 21 per cent sodium sulfate *B*, 0.5 per cent chromic ammonium sulfate *C*, 0.39 per cent crystalline cobaltous sulfate *P*, 0.00088N potassium permanganate

The concentration of protein was calculated from the standard curves which are described by the following equations, in which *D* is the optical density at 540 millimicrons

Total protein in serum	$\frac{D}{0.088} \times 100 = \text{per cent}$
Methyl alcohol filtrates	$\frac{D \ 0.004}{0.1145} \times 100 = \text{per cent}$
Sodium sulfate filtrates	
21 per cent*	$\frac{D \ 0.01}{0.072} \times 100 = \text{per cent}$
26 per cent†	$\frac{D}{0.0694} \times 100 = \text{per cent}$
15, 16, and 19 per cent†	$\frac{D + 0.012}{0.073} \times 100 = \text{per cent}$

\*Concentration of the final mixture obtained by adding 0.5 ml of serum to 9.5 ml of sodium sulfate reagent

†Concentration of the final mixture obtained by adding 0.5 ml of serum to 10 ml of sodium sulfate reagent



The filtrate from the method of Pillemer and Hutchinson with methyl alcohol was added at refrigerator temperature and the standard curve was prepared by also adding samples of filtrate at this temperature. The sodium sulfate filtrates were added at incubator temperature but the determination in 21 per cent salt

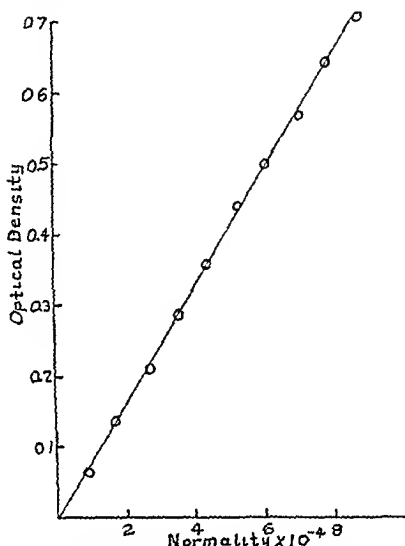


Fig. 2.—Values of optical density for different concentrations of potassium permanganate

was also made at room temperature. Standard curves were prepared at both temperatures and were found to be essentially the same when the concentration of the reagents of sodium sulfate was adjusted either to room temperature or to 37° C.

Although the temperature of the room is not critical, the results may be affected significantly in warm weather. Table I shows the values of optical density in a series of identical samples which were determined in a water bath at 25° and 30° C. During the summer months it is our practice to keep the tubes in a water bath at 25° until measurement in the colorimeter.

A final remark may be made concerning the determination of protein by the biuret reaction. Our experience confirms that of Wokes and Still that the intensity of the biuret color may be markedly increased in aged samples of serum.<sup>9</sup> We occasionally have found an apparent concentration of as much as 10 and 11 per cent in samples which were stored in the refrigerator for about six weeks. We do not have an explanation for the change which takes place, but it is probably due to bacterial action. There was no increase in the apparent concentration of normal serum that was drawn and stored in the refrigerator for

TABLE I MEASUREMENT OF THE BIURET COLOR IN SERUM AT 25° AND 30° C

SAMPLE	OPTICAL DENSITY		APPARENT CONCENTRATION	
	25°	30°	25°	30°
	D		GM PER 100 ML	
1	0.653	0.683	7.42	7.76
2	716	739	8.14	8.40
3	520	530	5.91	6.02
4	602	620	6.84	7.05
5	615	638	7.00	7.25
6	629	663	7.15	7.54
7	615	634	7.00	7.21
8	611	638	6.95	7.26
9	602	620	6.85	7.05
10	699	716	7.95	8.14
11	611	634	6.95	7.21

six weeks under sterile conditions. However, an apparent change from 7.5 to 8.5 per cent protein took place in the same serum when the flasks were opened occasionally without sterile precautions.

#### SUMMARY

A biuret reagent prepared according to Kingsley with 11.7 per cent sodium hydroxide is shown to be stable and is recommended for the determination of protein in serum and in both methyl alcohol and sodium sulfate filtrates of serum. Absorption curves are presented for the biuret color obtained with serum and with a sodium sulfate filtrate of serum, and for chromic ammonium sulfate, cobalt sulfate, and potassium permanganate. The curve of the latter most closely resembles that of the biuret color, and this substance is recommended for the standardization of the reagent.

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# STUDIES OF THE DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS

## I EXPERIMENTAL PERITONITIS

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A PREVIOUS report on patients with liver disease<sup>1</sup> established definite differences in the results of determinations of serum proteins by biuret and Kjeldahl methods. A further comparison of these methods was made in experimental peritonitis in dogs to confirm previous findings in similar diseases in human individuals. As indicated previously it is not known whether the changes observed represent a gain or a loss of nitrogen or of peptide linkages. For convenience they are described in terms of net change of biuret reactivity.

### METHODS

All protein determinations in this investigation were made simultaneously by the macro Kjeldahl and biuret methods,<sup>2</sup> using the ether sodium sulfate method<sup>3</sup> for the separation of albumin and globulin. Fresh unhemolyzed serum from blood samples taken either by vein or cardiac puncture was used. Adult mongrel dogs weighing an average of 16 kilograms were used. Prior to operation, small doses of morphine and atropine were administered subcutaneously. Ether anesthesia and aseptic technique were employed. Spreading peritonitis was induced according to the procedure described by Bower, Burns, and Mengle.<sup>4</sup> The appendix and its mesentery together with all its blood vessels, were ligated and the appendix was traumatized. Sixty to 90 ml of castor oil varied according to the weight of the animal, were administered by a catheter immediately following operation. Water was withheld for forty eight hours to avoid persistent vomiting. Simple laparotomies were performed on control animals exposing the viscera without ligation of blood vessels or traumatization of the appendix following the same anesthetization as in the experimental animals. Plasma volume and blood volume were estimated according to the method outlined by Gibson and Evans.<sup>5</sup>

### EXPERIMENTAL

Induced spreading peritonitis in dogs apparently caused an average decrease of biuret reactivity of globulin of 0.66 Gm per cent (Table I) with a standard error of  $\pm 0.08$ . This value is highly significant. Nine out of ten animals showed a significant difference in serum globulin as determined by biuret and Kjeldahl methods. Apparently the change occurs soon after the development of peritonitis since this difference was fairly uniform from the

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TABLE I DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS BEFORE AND 2 TO 10 DAYS AFTER SIMPLE LAPAROTOMY IN 3 DOGS AND SPREADING PERITONITIS IN 10 DOGS

DIFFERENCE, KJELDAHL MINUS BIURET						
	BEFORE OPERATION		AFTER OPERATION			
	ALB (GM %)	GLOB (GM %)	ALB (GM %)	GLOB (GM %)		
Standard deviation, laparotomy	-0 13	0 17	0 13	-0 13		
Standard deviation, peritonitis	0 07	0 06	0 04	0 66		
2 6 × std dev, laparotomy	±0 34	±0 44	±0 34	±0 34		
2 6 × std dev, peritonitis	±0 18	±0 16	±0 10	±1 72		
Number of dogs with changes in serum albumin and globulin after operation greater than 2 6 times the standard deviation (elevated, +, no significant change, 0, reduced, -)						
	ALBUMIN			GLOBULIN		
	+	0	-	+	0	-
Laparotomy	0	3	0	0	3	0
Peritonitis	1	8	1	9	1	0

second to the tenth day following the operation. No significant change occurred in the albumin biuret reactivity or total N during the same period. Average differences between protein determined by biuret and Kjeldahl N methods in both albumin and globulin were within normal limits before peritonitis was induced, and similarly, average differences in the control (laparotomy) animals were not significant. In spite of a large loss of 8 to 31 per cent (18.9 per cent average) of total circulating plasma protein, an increase of 0.4 Gm per 100 ml was observed in total protein by the Kjeldahl determination, probably as a result of dehydration. However, the biuret determination indicated no change in total protein before and after peritonitis. The Kjeldahl determination indicated a decrease in A/G ratio from 1.66 to 1.09, while a smaller decrease from 1.67 to 1.39 in A/G ratio was shown by the biuret method.

#### DISCUSSION

It has been shown<sup>1</sup> that liver disease will cause changes in the normal relationships between total N and biuret N in serum proteins, especially globulin. The degree of loss of biuret N in globulin in experimentally induced peritonitis is similar to that observed in cirrhosis. A comparable change in experimental peritonitis after two to ten days may be due in part to the rapid loss of large amounts of circulating plasma protein which placed an additional burden on a liver already actively involved and further impaired by the toxemias of the disease. Since these dogs ate little during the acute phase of the infection, malnutrition may have been an additional factor.

#### SUMMARY

1. Induced spreading peritonitis in dogs produced differences in serum globulin determinations by the biuret as compared with total nitrogen methods. These differences were similar to those observed in cirrhosis.

2. No corresponding changes in the albumin fraction were observed.

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# STUDIES OF THE DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS

## II EFFECT OF OCCLUSION OF THE HEPATIC ARTERY AND LIGATION OF THE GASTRODUODENAL ARTERY ON SERUM PROTEINS

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A PREVIOUS report on patients with various diseases<sup>1</sup> and one on animals<sup>2</sup> established definite differences in the results of determinations of serum proteins by biuret and Kjeldahl methods. Further comparison of these methods was made following occlusion of the hepatic artery and ligation of the gastroduodenal artery in dogs to determine the effect of a disturbance of arterial circulation and nutrition of the liver upon the biuret and Kjeldahl N relationship in serum proteins and in addition to establish the relationship of this liver impairment to previous findings in liver disease.

### METHODS

All protein determinations were made simultaneously by the macro Kjeldahl and biuret methods.<sup>3, 4</sup> The ether sodium sulfate method<sup>5</sup> was used for the separation of albumin and globulin. Only fresh unhemolyzed serum was used for all determinations.

Sodium Amytal intraperitoneally was used for anesthesia. A partial clamp was applied to the hepatic artery which was gradually tightened from one to eight minutes during the two or three weeks following the operation on three dogs. Cellophane was placed and held by two to three Michel skin clips around the hepatic artery to produce occlusion in fourteen other dogs. The gastroduodenal branch of the hepatic artery of all dogs was double ligated and divided at the time of operation. Ten or more weeks later the hepatic artery was ligated at the point of occlusion. Repeated determinations of serum proteins (biuret and total N), urea, nonprotein nitrogen, uric acid, hemoglobin, sugar, cholesterol, and cholesterol ester were made on the blood of the dogs for periods up to 120 days following the initial operation.

### RESULTS

In every dog in the periods studied (Table I) a loss of biuret reactivity occurred in the globulin fraction. The average loss of biuret N in the globulin fraction from the second to the one hundred and thirtieth day after the operation was 0.36 Gm per cent (Fig 1, Table I).

There was a gradual return to normal in the biuret N/Kjeldahl N ratio of the globulin following the operation from a loss of 0.40 Gm per cent biuret N to 0.08 Gm per cent on the thirty-fifth day. Losses occurred again until 0.58 Gm per cent globulin biuret N had been reached on the fifty-fifth day, after which a slow recovery occurred until the one hundred thirtieth day when a smaller loss of biuret N of 0.30 Gm per cent was reached. No average significant gain or loss in albumin biuret N occurred in the 130 days following the

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operation (Table I). However, the albumin values represented diagrammatically in Fig 1 show an irregular loss of albumin biuret N in the first thirty five days following the operation from 0.1 Gm per cent to -0.20 per cent

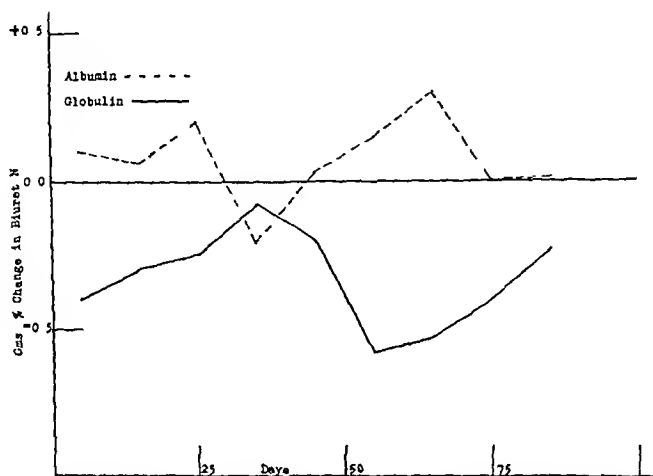


Fig 1.—Diagrammatic representation of gain and loss of biuret linkages (Gm % N) of serum proteins in dogs after occlusion of the hepatic artery and ligation of the gastroduodenal artery (data from Table I)

TABLE I DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS FOLLOWING OCCLUSION OF THE HEPATIC ARTERY AND LIGATION OF THE GASTRODUODENAL ARTERY

NUMBER OF DOGS	DAYS AFTER OPERATION	BIURET		DIFFERENCES BIURET MINUS KJELDAHL	
		ALB (GM %)	GLOB (GM %)	ALB (GM %)	GLOB (GM %)
10	2	3.7	2.1	0.1	-0.4
7	3 10	3.3	2.2	0.1	-0.4
11	11 30	3.0	3.4	0.1	-0.3
10	31 50	2.6	3.2	-0.1	-0.1
8	51 70	3.0	2.9	0.2	-0.0
13	71 90	2.9	3.0	0.0	-0.4
8	91 130	3.2	3.1	0.0	-0.3

Albumin biuret N increased from the thirty fifth day to the sixty fifth day when a gain of 0.30 Gm per cent was shown. The biuret N/Kjeldahl N ratio returned to normal on the seventy fifth to eighty fifth days. The greatest average loss of albumin biuret N occurred on the thirty fifth day. There was an apparent relationship between the simultaneous gain of albumin biuret N and loss of globulin biuret N, and loss of albumin biuret N and gain of globulin biuret N as shown in Fig 1. The highest globulin biuret N occurred at the

time of the greatest loss of albumin biuret N, and the reverse occurred when the greatest loss of globulin biuret N was reached at the approximate time of the greatest increase in albumin biuret N. A few animals, Dogs 405 and 427 (Fig 2) and Dogs 479 and 723 (Fig 3), showed marked reversal in their loss and gain of biuret N in albumin and globulin. A gain of globulin biuret N

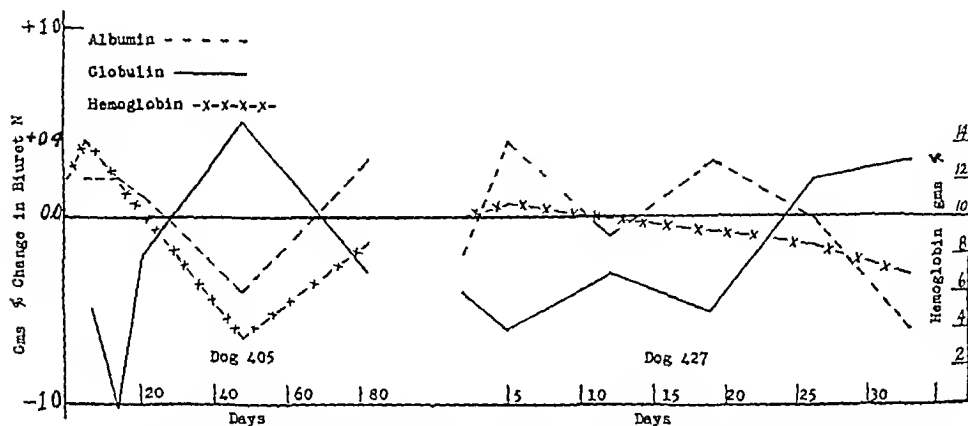


Fig 2—Diagrammatic representation of gain and loss of biuret linkages (Gm % N) of serum proteins and hemoglobin in dogs after occlusion of the hepatic artery and ligation of the gastroduodenal artery (data from Table I)

was reflected in a simultaneous loss of albumin biuret N, and loss of globulin biuret N occurred when there was a gain of albumin biuret N. This direct relationship was continuous throughout all periods studied after the dogs were operated upon.

Hemoglobin levels had a direct quantitative relationship to the biuret changes in albumin and globulin, as shown in Dogs 405 and 427 (Fig 2). Loss of albumin biuret N accompanied loss of hemoglobin, and an increase in hemoglobin occurred with a gain in albumin biuret N. However, only these two dogs had abnormally low hemoglobin levels.

All dogs showed no significant change in whole blood urea N, nonprotein nitrogen, uric acid, glucose, and serum cholesterol and ester. Only a few dogs showed slightly lowered hemoglobin levels.

All dogs were examined at autopsy, and sections of the liver, spleen, and arteries were taken. Histologic findings are to be reported elsewhere. The hepatic artery was completely occluded except in three dogs in which occlusion was almost complete.

#### DISCUSSION

The most persistent change in the serum proteins of dogs following occlusion of the hepatic artery and ligation of the gastroduodenal artery is the loss of globulin biuret N. This is also the most typical change in the serum proteins of patients with liver disease.<sup>1</sup> This similarity would indicate that impairment in arterial circulation and nutrition of the liver produces structural changes within a relatively short time in the serum proteins which are observed in cirrhosis after much more time has elapsed. The large loss of globulin biuret



N immediately after the operation in the dogs (Fig 1) probably results from the ligation of the gastroduodenal artery. Apparently collateral circulation to replace that of the gastroduodenal artery is established in the first thirty five days following the operation since the globulin biuret N returned to almost normal. However, there is a gradual loss of albumin biuret N during this time. This inverse relationship of the biuret N of albumin and globulin is

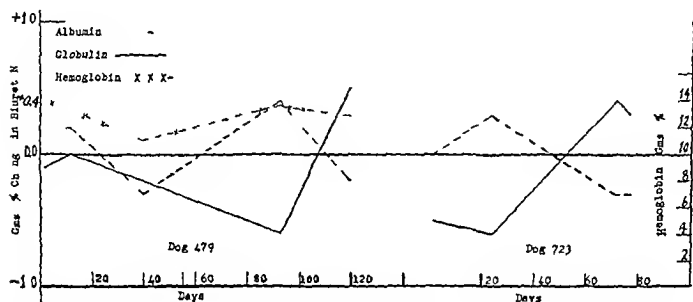


Fig 3—Diagrammatic representation of gain and loss of biuret linkages (Gm % N) of serum proteins and changes of hemoglobin in dogs after occlusion of the hepatic artery and ligation of the gastroduodenal artery (data from Table 1)

very clearly shown in individual Dogs 405 and 427 (Fig 2) and 479 and 723 (Fig 3). These changes observed in the thirty five days following the operation would primarily result from the malnutrition produced by the loss of gastroduodenal arterial circulation. The increasing loss of globulin biuret N accompanied by increase of albumin biuret N after the thirty eighth day following the operation (Fig 1) may have resulted from the gradual occlusion of the hepatic artery. This change increased until about the sixty fifth day when greater loss of globulin biuret N and gain of albumin biuret N were reached. The rate of occlusion of the hepatic artery was variable in the dogs. Some dogs examined showed complete occlusion of the hepatic artery after thirty five days. After the sixty fifth day the albumin and globulin biuret N again started to return to normal, probably as a result of establishment of collateral hepatic arterial circulation. Nearly every period studied in each dog showed a total net loss of biuret N. This is also true in induced peritonitis in dogs<sup>2</sup> in which the principle loss of biuret N occurs in the globulin. The tendency for the globulin fraction to be more labile to greater biuret N changes than albumin may be due to its larger molecular size which requires a more highly developed complex biologic process for its formation and maintenance. The biuret linkages in globulin may be less stable. The formation of globulin also may be secondary to that of albumin. However it is interesting that the approach to normal biuret N in albumin and globulin occurs at the same time (Figs 1, 2 and 3) and that their graph lines of a gain or loss of biuret N nearly always cross at 0.1 per cent biuret N. This would indicate that there is some definite relationship between

albumin and globulin in the mode of formation, origin, or exchange of their biuret N. There is also some indication that hemoglobin concentration has some relationship to gain and loss of biuret N in albumin and globulin (Dogs 405 and 427, Fig. 2). The greatest loss of albumin biuret N and the greatest gain of globulin biuret N is accompanied by the lowest hemoglobin levels. Since loss of albumin biuret N usually occurred only when the A/G ratio was 1 or less, it would appear that quantitatively albumin and globulin levels have some relationship to the maintenance of normal biuret N/Kjeldahl N ratio of serum proteins.

#### SUMMARY

1. Disturbance of the metabolism of the liver either by occlusion of the hepatic artery or ligation of the gastroduodenal artery in dogs is followed by a large loss of serum globulin biuret N and a smaller gain of serum albumin biuret N, resulting in a net loss of biuret N in the serum proteins.

2. No significant change in whole blood urea N, nonprotein nitrogen, ureic acid, glucose, and serum cholesterol and ester was observed in dogs following occlusion of the hepatic artery and ligation of the gastroduodenal artery. Low hemoglobins were observed in a few dogs.

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# STUDIES OF THE DIFFERENCES BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS

## III LIVER AND OTHER DISEASES

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**P**RELIMINARY reports on patients with chronic liver disease,<sup>1</sup> peritonitis in dogs,<sup>2</sup> and occlusion of the hepatic artery and ligation of the gastroduodenal artery in dogs<sup>3</sup> established definite differences in the results of determinations of serum proteins by biuret and total nitrogen (Kjeldahl) methods. Since good agreement between these methods exists in normal individuals,<sup>4</sup> further comparison was made in order to ascertain the significance and possible diagnostic value of such differences in various diseases. The changes observed indicate that either gains or losses of nitrogen or of biuret linkages have occurred. The data at present do not permit a distinction to be made.

### METHODS

All protein determinations in this investigation were made simultaneously by the macro Kjeldahl (using factor 6.25 to convert N to protein) and biuret methods.<sup>4, 5</sup> The ether sodium sulfate method<sup>6</sup> was used for the separation of albumin and globulin. Only fresh and unhemolyzed serum was employed. Urea and nonprotein nitrogen determinations were made to correct the Kjeldahl protein determinations for nonprotein nitrogen. The Kjeldahl digestion for total protein determination was carried out by gently boiling in a 300 ml Kjeldahl flask 10 ml of concentrated sulfuric acid, 4 Gm of sodium sulfate and 0.5 Gm of copper sulfate with 1 ml of serum for one hour. Twelve milliliters of albumin sodium sulfate solution from the globulin precipitation equivalent to 0.8 ml of serum were used for the albumin determination. The same amounts of reagents were used as in the total protein determination with the exception of the addition of only 1.5 Gm of sodium sulfate. No increase in total nitrogen was observed when digestion was continued for two, four and six hours.

### RESULTS

The data obtained from the study of various types of liver disease have been classified (Table I) as Laennec's cirrhosis, Banti's syndrome, biliary or obstructive, and miscellaneous hepatomegalias. The data of this table confirmed previous observations<sup>7-11</sup> with regard to decreased albumin and correspondingly decreased A/G ratio. Differences between the results of biuret and Kjeldahl determinations in the various types of liver disease were very similar. There was a consistent decrease of peptide linkages as measured by the biuret reaction (or increase in total nitrogen) of the globulin fraction in all types of liver diseases studied. The average loss of normal biuret reactivity of the globulin fractions of the eighty-four patients with liver disease studied was 16 per cent. The

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TABLE I DIFFERENCE BETWEEN BIURET AND KJELDAHL DETERMINATIONS OF SERUM PROTEINS IN VARIOUS HUMAN DISEASES

CLINICAL CONDITION	ALBUMIN				GLOBULINS			
	MEAN		STANDARD ERROR	NUMBER OF INDIVIDU- ALS WITH KJELDAHL MINUS BIURET N GREATER THAN T*	MEAN		STANDARD ERROR	NUMBER OF INDIVIDU- ALS WITH KJELDAHL MINUS BIURET N GREATER THAN T*
	BIURET (OM %)	KJELDAHL (OM %)			BIURET (GM %)	KJELDAHL (OM %)		
	5.25	5.28	±0.02	+ 0	2.08	2.13	±0.04	+ 0
Normal <sup>a</sup>				0				0
Diseases of liver				15				15
Laennec's cirrhosis	3.80	3.72		2	2.78	3.26		4
Banti's syndrome	3.90	3.68		0	2.30	2.92		1
Gall bladder disease	4.00	3.98		5	2.55	3.04		6
Liver enlargement	4.31	4.30		3	2.33	2.72		7
Carcinoma	3.81	3.80		0	2.58	2.79		3
All liver diseases	4.00	3.96	±0.12	10	2.53	3.00	±0.10	21
Thyroid disease	4.48	4.37	±0.05	1	2.11	2.54	±0.24	4
Diseases of blood	4.23	4.15	±0.26	3	2.95	2.74	±0.39	5
Typhoid fever	3.75	3.73		0	2.55	3.03		4
Typhoid recovered	4.50	4.58		0	2.55	2.68		1
Miscellaneous diseases				13				19
				29				18
				9				14

\*T =  $\frac{\text{Average difference}}{\text{Standard error}}$  where P = 0.01

average difference of 0.47 grams per cent between protein determined by Kjeldahl and biuret methods in the globulin fraction was highly significant as the standard error of this difference was only  $\pm 0.10$

This difference between Kjeldahl and biuret determinations of the globulin fraction was reflected in a corresponding difference of Kjeldahl and biuret N in the total protein determinations which resulted in a significantly higher A/G ratio as determined by the biuret method. On the average, there was no significant increase in the biuret reactivity of the albumin, although there appeared to be a tendency for an increase in twenty-four cases. In the group of eighty-four cases of hepatic disease only three—one of chronic cholangitis, one of scurvy with hepatomegaly, and one of metastatic neoplasm, exhibited a significant increase in biuret linkages of the globulin fraction.

Toxic thyroid disease (Table I) was very similar to portal cirrhosis in the abnormal biuret reactivity/Kjeldahl N ratio of the globulin fraction. A decrease of 17 per cent in the biuret linkages of the globulin fraction occurred. However, as the number of cases in this group was small, the standard error of the differences of the Kjeldahl and biuret determinations of globulin and A/G ratio was not as highly significant as in the much larger group of liver diseases. The increase of biuret linkages of the albumin fraction even though small was much more significant than that found in portal cirrhosis. The total protein and A/G ratio as determined by the Kjeldahl method were fairly normal, the A/G ratio being the highest of any group studied. This demonstrates that in disease, serum proteins may be normal quantitatively by Kjeldahl determinations when they are actually abnormal in their biuret linkages. The finding of a small significant change in the albumin fraction in thyrotoxicosis together with a large loss of biuret linkages in the globulin fraction substantiates the evidence<sup>12, 13</sup> presented for the activity of the thyroid in the formation of serum globulin. However, secondary liver damage may follow thyroid hyperactivity.

A majority of the various types of anemias (diseases of the blood (Table I)) as individual cases showed an increase in biuret linkages in both protein fractions. This group on the average showed no significant increase in biuret linkages, probably because of the great range of individual values (globulin 1.4 to 5.4) and because of the different types of anemias in the small group. However, if the gain and loss of biuret linkages in serum albumin and globulin are added, a significant net gain in biuret linkages is observed, the average gain being 0.3 Gm per cent with a standard error of  $\pm 0.12$  per cent. This is the only group studied which showed an overall gain in biuret linkages. These data indicate that a disturbance of the blood cells may act as a stimulus for the temporary overproduction of biuret linkages in serum albumin and globulin.

Serial determinations of biuret linkages (Table I) were made in four cases of typhoid fever from the second and fourth to the eighth and twelfth week of the disease. Little change occurred in the albumin fractions except in one case complicated by hypertension. In all, the globulin fraction showed a loss of biuret linkages starting with the fourth week and returned to normal with clinical recovery. The loss of biuret linkages in globulin resembled that in

portal cirrhosis and may indicate at least temporary liver damage in typhoid. An indication also was given that the liver's ability to form normal proteins was noticeably impaired until after the third week of illness.

A large number of miscellaneous diseases, some of which showed significant changes in the distribution of biuret linkages in the protein fractions, are summarized in Table I. A case of malnutrition with transfusion reaction and another transfusion reaction showed a marked loss of biuret linkages in the albumin fraction. Diabetes, diabetic coma, and complicated diabetes showed wide variations in distribution of biuret N. Two cases of cystinuria showed a simultaneous loss of biuret N in the globulin and gain in the albumin fractions. A case of chronic ulcerative colitis resembled malnutrition in biuret N changes as did nutritional edema. A patient with myxedema showed a marked increase in biuret N in the globulin with no change in the albumin. This is in contrast to the decrease in biuret N in the serum globulin of thyrotoxicosis. A patient with dystonia quadriplegia showed an almost equal exchange of biuret N from globulin to albumin, a loss and gain of about 20 per cent. A case of schizophrenia showed an opposite transfer. However, this patient was suspected of having hepatic disease, although no definite diagnosis could be made. One patient with lymphogranuloma inguinale, who showed marked inversion of A/G ratio, exhibited changes in biuret N similar to those in portal cirrhosis. In one patient with lymphopathia venereum there was an increase in biuret linkages of the globulin fraction, while two others showed a decrease, all three of these patients had definite hyperglobulinemia. Patients suffering from cardiac decompensation, acute nephritis, myoma uteri, and bronchopneumonia showed no changes in the normal distribution of biuret N.

#### CASE REPORTS

A few interesting cases exhibiting reversal of biuret and Kjeldahl N differences coinciding with marked clinical changes were studied over a period of several weeks. A colored man, 30 years old (No. 1, Table II), with an apparently quiescent sickle cell anemia showed about 20 per cent gain in biuret linkages of the globulin fraction. There was also a 16 per cent loss of biuret linkages in the albumin fraction. About two weeks later, the distribution of biuret reactivity in the protein fractions had returned to normal. However, this condition was not stabilized. A marked reversal of biuret distribution occurred four weeks later resulting in a 26 per cent loss of biuret reactivity in the globulin fraction and a small increase of biuret N in the albumin fraction. During these six weeks the icterus index had increased from 7 to 450, urea N from 14 to 23 mg per 100 cc, while cholesterol ester decreased to 1 per cent of the total cholesterol. Although the total protein was elevated with a low A/G ratio, of 0.7 to 0.8, there was little significant quantitative change in the protein fractions throughout the six week period. This again shows that marked qualitative changes in the proteins may take place while no quantitative changes occur.

A 6 year old girl, five months after second degree burns (No. 2, Table II) exhibited changes in biuret reactivity of serum proteins similar to those observed in portal cirrhosis. Six to eight weeks later, coincident with malnutrition, fever, anemia, and urinary tract infection, a reversal of biuret nitrogen distribution had occurred with a loss in albumin and a gain in globulin biuret linkages. Eleven weeks later after a diet rich in amino acids, proteins and vitamins, the patient showed marked clinical improvement and the biuret distribution had returned to normal. However, seven weeks later the patient returned to the hospital with malnutrition, fever, and anemia and again exhibited abnormal biuret distribution typical of this clinical change.

TABLE II REVERSAL OF KJELDAHL MINUS BIURET DIFFERENCES IN DISEASED INDIVIDUALS

NO	CLINICAL CONDITION	CHANGES IN KJELDAHL PROTEIN MINUS BIURET PROTEIN (GM %)							
		WITH TIME							
1	Sickle cell anemia	Albumin	0.5	0.0	0.2	0.5			
		Globulin	-0.9	-1.0	1.2	0.2			
		Date	3/10	3/26	4/23	5/1			
2	Burns	Albumin	0.3	-0.5	0.7	0.3	0.9		
		Globulin	0.7	0.2	-0.2	0.2	-0.3		
		Date	3/25	4/10	4/23	7/11	9/27		
3	Multiple myeloma	Albumin	0.3	0.0	0.2	0.2			
		Globulin	-0.4	0.6	0.3	0.8			
		Date	7/11	7/14	9/25	10/9			
4	Biliary cirrhosis	Albumin	0.0	-0.1	0.1	0.1			
		Globulin	1.2	1.4	1.0	1.5			
		Date	10/7	10/21	11/7	11/25			
5	Nephrotic syndrome	Albumin	0.2	0.7	0.6	0.2	0.5	0.4	0.2
		Globulin	0.2	0.5	-0.4	0.0	-0.2	-0.6	0.0
		Date	5/12	5/14	5/15	5/16	5/20	5/21	6/4

A patient with multiple myeloma with pathologic fracture of the femur (No 3 Table II) and a large increase in globulin showed an increase in biuret linkages in the globulin fraction following the fracture. Three months later, a large decrease in biuret reactivity in the globulin occurred. Qualitatively and quantitatively the albumin remained fairly constant throughout the period of investigation. It is interesting to note that a quantitative globulin increase was accompanied by an increase in biuret reactivity in this case and a fall of total globulin to normal level was accompanied by a decrease in biuret reactivity.

No 4 (Table II) illustrates in biliary cirrhosis the constancy with which a large loss of biuret reactivity occurred in the globulin fraction over a period of several weeks with little change in albumin. Administration of bile salts for twenty days had no apparent effect on the proteins.

Simultaneous biuret and Kjeldahl determinations were carried out before several plasma transfusions in a patient presenting the symptoms of a nephrotic syndrome (No 5, Table II). Although the control for biuret linkages of albumin and globulin serum fractions before the first transfusion was within normal limits the biuret linkages of the albumin decreased and those of the globulin increased twenty three to thirty hours following transfusion. The net change as shown in total protein biuret reactivity was within normal limits in nearly every series of analysis. Two weeks following the last transfusion the biuret linkages of both serum albumin and globulin fractions had returned to normal.

#### DISCUSSION

Considerable evidence has been presented in the literature to show that qualitative as well as quantitative changes occur in serum proteins in various diseases. Analysis of the data presented in Table I has shown that very significant changes occur in the relationship between biuret linkages and total nitrogen of serum globulin during liver disease. A difference in the distribution of serum globulin fractions in normal and cirrhotic individuals has been shown by electrophoretic and immunologic means<sup>14</sup>. There is much evidence that fibrinogen production is wholly dependent upon liver function. The role of the liver in the production of albumin and globulin is not as well established. There is however clinical and experimental evidence that the organ is involved in the processes of albumin and globulin formation especially the former. Whipple and co workers<sup>15, 16</sup> noted a lag in the regeneration of serum proteins of Eck fistula dogs following acute plasma depletion. Hepatectomy experiments in various animals have added little significant evidence relating to the origin and

mode of formation of plasma albumin and globulin since such a diastic procedure gives rise to profound systemic and circulatory disturbances. Hypoproteinemic dogs<sup>17</sup> exhibited a decreased deamination in impaired livers.

A relative loss of biuret linkages of serum globulin together with a concurrent gain of biuret linkages of albumin in toxic thyroid disease may indicate that the thyroid has some influence on formation, loss, or exchange of biuret linkages in serum proteins. Since no significant increase in biuret linkages of serum albumin occurred in liver disease, these alterations of serum albumin in thyroid disease cannot be explained as a result of secondary liver disease.

The tendency for overproduction of biuret linkages of both albumin and globulin in some anemias studied (Table I) may indicate a disturbance of a part of the normal function of blood cells in the formation of serum proteins. Hyperproteinemia observed in myeloid leucemia may indicate that serum globulin has its origin in bone marrow.<sup>18</sup> Since plasma proteins are within normal limits in aplastic anemia, more evidence is necessary to establish the proper function of marrow cells in protein formation.<sup>19</sup> However, the globulin albumin ratios for aplastic anemia are above normal.<sup>20</sup> In No. 1 (Table II), during sickle cell anemia, an enormous overproduction of biuret linkages equal to 1 gram per cent protein N in the globulin fraction occurred. In multiple myeloma, No. 3 (Table II), a large increase in biuret linkages in the serum globulin occurred in the early acute stage of the disease when there was probably an overstimulation of the red marrow. Two to three months later, a decrease of biuret linkages in serum globulin was observed. This probably was caused by the impaired activity of either the red marrow or liver. The production of a serum protein greatly altered in normal structure may be an important factor in the origin of the abnormal Bence Jones protein of multiple myeloma. Loss of biuret linkages of the globulin fraction in blood serum during chronic febrile disease similar to that observed in liver disease probably results from secondary liver damage caused by a fever of long duration. From a study<sup>20</sup> of the electrophoretic patterns of a series of normal and pathologic human sera, it was found that the serum  $\alpha$ -globulin albumin ratios were high in febrile patients, the average value being over twice the normal. This was particularly marked in pneumonia.<sup>21, 22</sup> Polarographic study<sup>23, 24</sup> of blood sera in experimental pneumonia (Type 1) in dogs has shown a decrease in wave heights in the denaturation curves which represented some change in serum (peptone fraction), probably a decrease in protein sulphydryl and disulfide groups, i.e. a decrease in cystine in peptide linkage. In acute infections, a protein has been found in the albumin fraction which gave a flocculation reaction with C polysaccharide of pneumococcus.<sup>25</sup> Electrophoresis has shown a decreasing albumin and increasing globulin in tuberculosis with the appearance of a new X component.<sup>26</sup>

The reversal of biuret and total nitrogen ratio in serum protein (Table II) over a comparatively short period of time in some diseases may indicate some relationship between albumin and globulin as to their interdependence, common origin, and mode of formation. It will be noted from the diagrammatic representation (Fig. 1) of the simultaneous gain and loss of biuret linkages in serum



albumin and globulin of Nos 1 2 and 3 presented in Table II that there is a definite relationship between albumin and globulin as to their relative gain or loss of biuret linkages. In No 2, especially, it is apparent that a gain or loss of biuret linkages in albumin is matched by an equal change in the opposite direction in the globulin. In all three cases an approach to normal in biuret

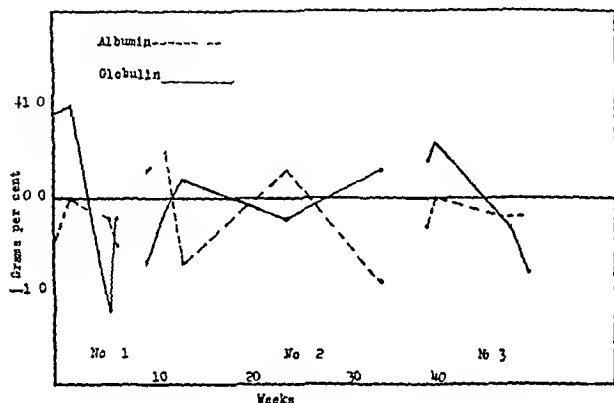


Fig 1—Diagrammatic representation of simultaneous gain and loss (grams per cent N) of biuret linkages in serum albumin and globulin (data from Table II)

linkages in both albumin and globulin occurs at the same time. In studies of protein metabolism by the isotope method<sup>27</sup> synthesis and degradation were found to be a balanced biochemical process which requires no distinction between exogenous and endogenous cellular metabolism. Labile protein reserves have been demonstrated<sup>28</sup> and may pass readily from cell to plasma and plasma to cell<sup>29</sup>. A depletion of these reserves lowered resistance to infection<sup>30</sup> and increased susceptibility to liver injury<sup>31, 32</sup>. These reserve protein stores have not been identified chemically<sup>33</sup>. The interchange of biuret linkages between albumin and globulin indicates the possibility of a normal exchange of biuret N between albumin and globulin, and/or they have a common origin in a labile protein reserve.

In most of the diseases studied it appears that the biuret linkages of the globulin are more labile and undergo wider variations than those of albumin. Liver typhoid, and thyroid diseases show a significant loss of biuret linkages in serum globulin. In these diseases the biuret exchange of a precursor type of albumin with globulin may be arrested. This also may be related to the greater molecular size of the globulin molecule.

It is interesting to note the changes in biuret linkages of serum proteins in a case of nephrotic syndrome (23 to 32 Gm protein loss per twenty four hours) (Table II) twenty three to thirty hours after a transfusion of 300 to

450 ml of plasma Total biuret linkages remain little changed while equally large losses in the albumin and gain in the globulin biuret linkages occur over a period of about four weeks A response of this kind would certainly indicate a lack of normal utilization of the transfused proteins This patient exhibited biuret linkages within normal limits in both albumin and globulin before and several days after the last transfusion The gaining of biuret linkages in globulin at the expense of albumin may be an indication that albumin acts as an intermediary labile protein or that the mechanism for the formation of albumin is repressed as might be expected where the A/G ratio is extremely low It is also interesting to note that the A/G ratios by biuret determination remained abnormally low and unchanged after transfusion, while by Kjeldahl determination the A/G ratios were normal in four instances

The possible diagnostic value of the determination of the ratio of biuret N to Kjeldahl N in serum proteins is obvious, since changes in ratio of biuret N to total N of serum proteins in various diseases show definite patterns and in some diseases Kjeldahl determinations indicate normal serum proteins when their biuret linkages are abnormal

Lack of agreement between determinations by the Kjeldahl and biuret methods may result from (1) a change in nitrogen concentration, (2) a change in number or reactivity of biuret linkages, (3) a change in response to salting out with sodium sulfate, or (4) a combination of (1), (2), and (3) These changes may be related to alterations in number, kind, or structural arrangement of amino acids in the protein molecule, or altered content of sugars, lipids, etc., forming part of the protein molecule Of the three possibilities, (1) seems more likely to be the cause of the lack of agreement of the two analytic methods since the biuret linkages are so numerous and fundamental in protein structure that rupture or replacement of these bonds by other types would probably result in greater physical changes in plasma or serum proteins than those observed in the conditions studied However, both (1) and (2) may be simultaneously operative or each may predominate under certain conditions Peters and Van Slyke<sup>34</sup> mention the altered chromogenic power of serum proteins in disease, in reference to the use of tyrosine as a standard in the Greenberg<sup>35</sup> serum protein method The possibility that polypeptides, amino acids, or other biuret reacting compounds were causing the differences observed was investigated in several cases where unusual differences between the biuret and Kjeldahl methods were noted The concentration of these compounds was never high enough in the plasma or serum to have any appreciable effect on the biuret reaction

Incomplete digestion of some amino acids of proteins have been reported,<sup>36</sup> but errors of this kind could not have occurred, since great care was exercised in all of our Kjeldahl determinations The Kjeldahl digestion technique used in this investigation agreed well with the method recommended in an exhaustive experimental survey of Kjeldahl methods<sup>37</sup> Our Kjeldahl-biuret analyses are not in agreement with a report<sup>38</sup> of significant differences of protein determinations on normal human serum made by the Kjeldahl and Kingsley biuret methods However, analyses for only four normal serums and one cirrhotic serum were reported

## SUMMARY

1 In various diseases, differences were observed in serum albumin and globulin determinations by the biuret and total N methods. In Laennec's cirrhosis, Banti's syndrome, biliary cirrhosis, hepatomegaly due to other causes, thyrotoxicosis, and typhoid fever, the principal change is loss of biuret linkages by the globulin.

2 In certain anemias an increase of biuret linkages in both albumin and globulin is the rule, although a rapid reciprocal change of biuret reactivity can take place in the protein fractions in some instances.

3 In general it may be said that primary hepatic disease or secondary liver damage resulting from impairment in circulation, malnutrition, fevers of long duration, severe infections, or tissue damage will cause changes in the normal relationship between total N and biuret reactivity of the serum proteins.

4 Theoretical implications derived from experimental clinical data as to the chemistry of certain phases of serum protein formation or degeneration as well as the actual chemical change responsible for abnormal biuret N and total N ratios have been presented.

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## HEBERDEN'S NODES THE RELATIONSHIP OF THE MENOPAUSE TO DEGENERATIVE JOINT DISEASE OF THE FINGERS

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HEBERDEN'S nodes are enlargements of the terminal interphalangeal joints of the fingers due to degenerative joint disease. Individual fingers are at times affected, especially in men, as a result of direct injury which produces a true traumatic arthritis. The deformities affecting multiple fingers on both hands, however, arise spontaneously without history of injury. The present study is confined to the latter type which is called idiopathic Heberden's nodes. Age is perhaps the most important single factor determining the appearance of this condition<sup>1</sup>. Heredity is also important, as is shown by vertical involvement in families in succeeding generations and horizontal involvement in siblings<sup>2</sup>. Women are affected much more commonly than men and, since Heberden's nodes are rare before the age of 50, their occurrence is thought to be related to the climacteric. The present study was made to test this supposition and to consider the mechanism by which the change of life influences the development of this deformity.

### CLINICAL OBSERVATIONS

Observations were made on ninety-nine women with idiopathic Heberden's nodes who had passed the menopause and who gave a reliable history of the time of occurrence of the menopause and appearance of the nodes (Table I). The age of these women at the time of the onset of Heberden's nodes ranged from 33 to 65 years. The median age was 49.8 years, the average age was  $48.8 \pm 6.80$  years. The age distribution at the time of onset of Heberden's nodes is shown in Fig. 1.

The age at the time of the menopause in this series of ninety-nine women ranged between 28 years, as the result of a pelvic operation, and 56 years. The median age was 49.7 years, the average age was  $48.6 \pm 5.25$  years. The age distribution is shown in Fig. 2.

The history of the menopause in these ninety-nine women with Heberden's nodes was compared with that of a control series of ninety-six women (Fig. 3). The control series had been assembled and observed in a previous study under the same conditions and at the same time as the Heberden's nodes series. The control series included the sisters of forty-three women chosen from the wards of City Hospital simply because they did not have Heberden's nodes, a collection of families observed in connection with the studies on heredity of Heberden's nodes. In the control series the age at which the menopause occurred

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<sup>2</sup>Read at the meeting of the American Rheumatism Association in Atlantic City, June 7, 1947.

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TABLE I TIME RELATIONSHIP BETWEEN HEBERDEN'S NODES AND MENOPAUSE, NINETY NINE CASES

CASE	PATIENT	AGE AT ONSET OF HEBERDEN'S NODES	AGE AT ONSET OF MENOPAUSE	INTERVAL (YR) *
1	Co	33	38	+ 5
2	Bl	33	53	+20
3	Ba	35	39	+ 4
4	Ra	37	46	+ 9
5	Je	38	28	-10
6	Go	39	39	0
7	Ac	39	50	+11
8	St	40	46	+ 6
9	Br	40	49	+ 9
10	Ra	41	40	- 1
11	He	41	41	0
12	Ra	41	43	+ 2
13	Fr	42	43	+ 1
14	Cr	42	42	0
15	Ch	42	40	- 2
16	Po	42	46	+ 4
17	Ji	42	48	+ 6
18	Ku	42	50	+ 8
19	Pa	43	43	0
20	Jo	43	46	+ 3
21	Le	43	52	+ 9
22	Ch	44	38	- 6
23	H1	44	44	0
24	Hu	44	40	- 4
25	Gr	44	48	+ 4
26	Al	45	47	+ 2
27	Mc	45	45	0
28	Am	45	46	+ 1
29	Br	45	50	+ 5
30	He	45	53	+ 8
31	Si	45	53	+ 8
32	Ha	46	47	+ 1
33	Mc	46	47	+ 1
34	Ho	46	56	+10
35	Li	47	47	0
36	Wa	47	48	+ 1
37	Fo	47	50	+ 3
38	En	47	50	+ 3
39	Du	47	52	+ 5
40	Br	48	33	-15
41	Wh	48	48	0
42	Be	48	49	+ 1
43	Ci	48	50	+ 2
44	Fi	48	50	+ 2
45	Pe	48	55	+ 7
46	Ba	49	35	-14
47	Sh	49	50	+ 1
48	Sm	49	51	+ 2
49	So	49	51	+ 2
50	Do	49	54	+ 5
51	Wa	50	44	- 6
52	Wr	50	45	- 5
53	Br	50	48	- 2
54	Vo	50	50	0
55	Ca	50	50	0
56	Co	50	52	+ 2
57	He	50	52	+ 2
58	Ve	50	52	+ 2
59	Th	50	56	+ 6

TABLE I—CONT'D

CASE	PATIENT	AGE AT ONSET OF HEBERDEN'S NODS	AGE AT ONSET OF MENOPAUSE	INTERVAL (YR) *
60	La	51	54	+ 3
61	Br	51	56	+ 5
62	Sm	52	42	-10
63	Ha	52	43	- 9
64	Bo	52	47	- 5
65	La	52	48	- 4
66	Sp	52	49	- 3
67	St	52	50	- 2
68	St.	52	53	+ 3
69	Br	53	49	- 4
70	Sa	53	50	- 3
71	Ca	53	51	- 2
72	Un	53	54	+ 1
73	Ha	53	55	+ 2
74	Jo	54	50	- 4
75	Mc	54	51	- 3
76	Be	54	52	- 2
77	Mc	54	50	- 4
78	Fa	55	50	- 5
79	Go	55	48	- 7
80	Ha	55	45	-10
81	Ca	56	50	- 6
82	Le	56	50	- 6
83	Mo	57	51	- 6
84	Po	57	52	- 5
85	Co	57	53	- 4
86	Po	57	55	- 2
87	El	57	56	- 1
88	Kr	58	47	-11
89	Mc	58	50	- 8
90	Ba	58	56	- 2
91	Yo	59	49	-10
92	Sm	59	52	- 7
93	Bi	59	54	- 5
94	Me	60	54	- 6
95	Pi	61	52	- 9
96	Mi	62	48	-14
97	He	62	48	-14
98	Fl	64	53	-11
99	Br	65	51	-14

The interval is given a plus sign (+) when the age at time of onset of the menopause was greater than the age at the onset of Heberden's nodes and a minus sign (-) when the reverse was the case

ranged from 35 to 59 years. The median age was 49.1 years, the average age was  $48.9 \pm 4.90$  years. The age distribution in the control series of women is shown in Fig. 3. Thus it is seen that the history of the menopause in ninety-nine women with Heberden's nodes paralleled closely that of ninety-six women who were used as controls. The history of the menopause in this group of ninety-nine women with Heberden's nodes was not considered to be remarkable.

The time relationship between the menopause and the onset of Heberden's nodes in each individual woman is shown in Fig. 4. The menopause preceded the onset of Heberden's nodes in forty-four instances and followed it in forty-five. Both events occurred in the same year in ten instances. The average difference in time between these two events regardless of which event occurred first was  $4.9 \pm 4.04$  years. If the time interval between these events

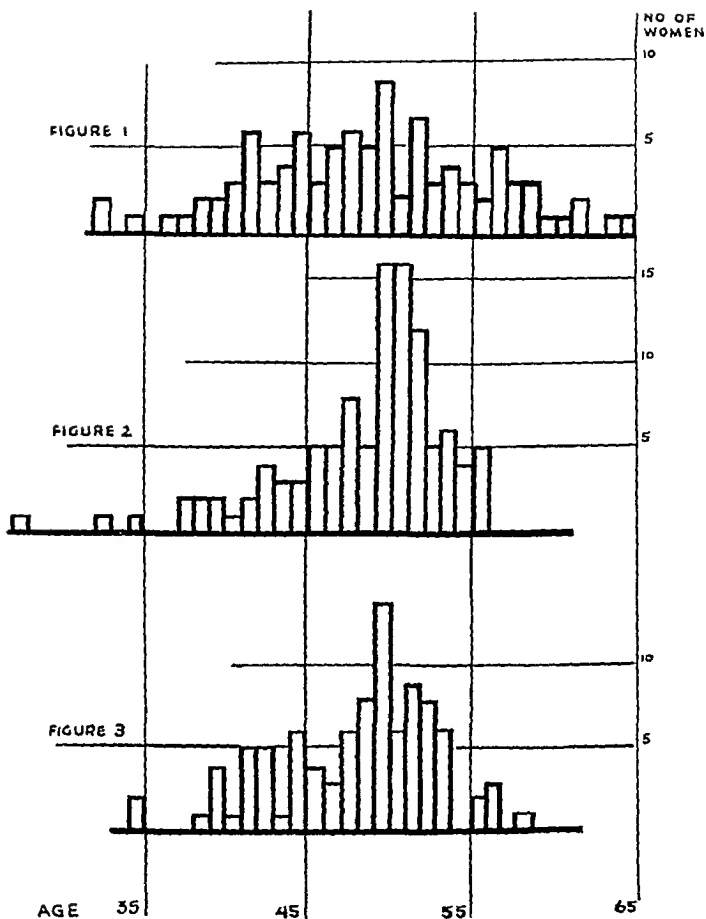


Fig 1 —Age at the onset of Heberden's nodes in the ninety-nine women studied  
Fig 2 —Age at the onset of the menopause in the ninety-nine women with Heberden's nodes  
Fig 3 —Age at onset of the menopause in ninety-six women of the control series

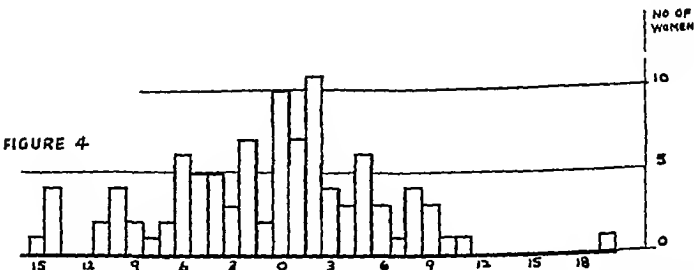


Fig 4 —Time relationship between the age at onset of Heberden's nodes and the onset of the menopause in individual cases. Zero represents the age at onset of Heberden's nodes. Figures to the left of zero indicate the years before onset of Heberden's nodes at which the menopause occurred. Figures to the right of zero indicate the years after onset of Heberden's nodes at which the menopause occurred.



was computed always in the same direction, applying a positive sign to the number of years difference when the menopause followed the onset of Heberden's nodes and a negative sign when the menopause preceded the onset of Heberden's nodes the average difference was  $-0.72 \pm 6.33$  years. The average difference practically disappeared and the two events seemed to be closely correlated in point of time. There was, however, a long time interval between these events in individual cases the menopause occurring from twenty years after the onset of Heberden's nodes to fifteen years before. This is indicated by the large value for the standard deviation.

Despite the wide dispersion, close association of the menopause with the onset of Heberden's nodes was observed in many cases. In ten instances both events occurred in the same year. In forty-four instances, or nearly half of the series, they occurred within three years of each other. The time relationship of these two events, independent of the age at which they occurred, was then studied. As has been shown already the average age of onset of Heberden's nodes in these ninety-nine women was  $48.8 \pm 6.8$  and the average age for the menopause was  $48.6 \pm 5.2$ . When the group was divided into two equal parts on the basis of age it was found that those women whose Heberden's nodes occurred early had an earlier onset of the menopause than those in whom the onset of the nodes was late. The average age of the menopause was 46.2 years in the fifty women having the earliest onset of Heberden's nodes at 33 to 49 years. The average age of the menopause was 50.5 years in the forty-nine women having later onset of Heberden's nodes at 50 to 65 years.

The degree of correlation between ages of onset and the menopause was calculated from grouped data by the usual product moment method for the determination of the coefficient of correlation ( $r$ ). The value of  $r$  with its standard error is  $+0.46 \pm 0.08$ . By using the test recommended by Peil<sup>3</sup> this coefficient of correlation can be regarded as arising by chance from an uncorrelated population about once in a million times. The result leaves no doubt that the data show a highly significant statistical association between the ages of onset of Heberden's nodes and the menopause.

While a correlation coefficient of  $+0.46$  is descriptive of the statistical association between the ages of onset of Heberden's nodes and the menopause it gives no reliable indication of the causal factors involved. On the basis of certain assumptions, it is possible speculatively to draw a further conclusion by analogy with the interpretation of better known cases. It happens that the coefficient of correlation between stature of father and son is  $+0.46$ . Assuming that the number of elements causing the variability is the same in both instances and that they are normally distributed the conclusion is drawn that about 46 per cent of the genes determining stature are common to parent and offspring of the same sex. It seems fair to make similar assumptions in the present instance. The data are consistent with this assumption although they do not prove it. A number of factors obviously are involved in causing the variability in ages of onset of Heberden's nodes and of the menopause. The average age and the median age are about the same for both variables, indicating an approximately normal distribution for the causal factors involved.

Consequently, if the assumptions are justifiable, the conclusion is drawn that, on the average, 46 per cent of the total set of factors determining age of onset are common to Heberden's nodes and the menopause.

#### LITERATURE

Many observers have noted a close relationship between the menopause and Heberden's nodes. Heberden<sup>1</sup> himself made no mention of the menopause in his very short description of "digitorum nodi" and neither did Charcot.<sup>2</sup> Haygarth,<sup>3</sup> about the beginning of the nineteenth century, stated that nodosities of the joints are almost peculiar to women and generally begin about the period when the menses naturally cease. Duckworth,<sup>7</sup> who thought Heberden's nodes were related to gout, said, "these may arise from the second to the fifth decade, are almost common perhaps as a gouty manifestation in women about or after the menopause." Monroe<sup>8</sup> stated that women display Heberden's nodes first about the menopausal period. Aschner<sup>9</sup> said he had seen Heberden's nodes often combined with the usual form of polyarthritis of the menopause. Cecil and Archer<sup>10</sup> noted Heberden's nodes in 74 per cent of the women in their series with menopausal arthritis. The *Primer on Rheumatism*<sup>11</sup> stated that Heberden's nodes often develop at the time of the menopause.

Besides the foregoing casual comments concerning the association of Heberden's nodes with the menopause, several students have presented statistical data to support the idea that the development of Heberden's nodes is related to ovarian dysfunction. Of forty seven women studied by Pineles,<sup>12</sup> thirty one noted the appearance of Heberden's nodes within a year of the last menstrual period. Four other women observed that their nodes occurred after the last menstrual period but while they were still having climacteric symptoms such as dizziness, nervousness, palpitation, hot flushes, and fleeting pains in various parts of the body. Four women under 45, though still menstruating regularly were having typical symptoms of the climacteric when their nodes began. Five women under the age of 40 stated that their nodes began shortly after pelvic operations which had resulted in cessation of the menses and menopausal symptoms. Only three of the forty seven women developed Heberden's nodes independent of the menopause or menstrual dysfunction. Von Noorden<sup>13</sup> described Heberden's nodes and other manifestations of degenerative joint disease in women under 30. In all such instances he had noted abnormalities of the sexual phenomena such as amenorrhea, sterility, or atrophic genitalia. Albright and co workers<sup>14</sup> described a 37 year old woman with ovarian agenesis who had well developed Heberden's nodes.

The clinical data seem to indicate a strong correlation between the climacteric and the development of Heberden's nodes. Since these deformities represent a particular manifestation of degenerative joint disease, it seemed pertinent to search the literature for evidence of correlation between the climacteric and this much broader and more general disease entity. The menopause is thought by some authors to cause or influence the onset of rheumatoid arthritis, by others to favor the development of osteoarthritis, while a third group believe its influence is limited to the production of arthralgia.<sup>15</sup> Hall,<sup>16</sup> studying seventy one women who had been ovariectomized, found most of them suffering from symptoms of arthritis beginning a few weeks after operation and often suggesting true rheumatoid arthritis. Eighteen of his patients had symptoms of either true rheumatoid arthritis or osteoarthritis. He thought fifty three of his patients had only arthralgia because no true joint disease developed.

The concept of arthropathia ovaripriva was discussed by Menge.<sup>17</sup> He observed a series of women having joint disturbances arising soon after inactivation of the ovaries by radiation. Complaints most often concerned the knees but frequently included the shoulder and occasionally the fingers. Involvement was most often bilateral. Crepitus on joint motion was frequently noted. Pain in some patients was slight but occasionally was severe enough to interfere with function. Menge was definitely of the opinion that the syndrome was due to ovarian failure, the same as occurred in spontaneous menopause.

Arthritis of the menopause was described by Cecil and Archer<sup>10</sup> as "a chronic polyarthritis of obese middle aged women occurring at or just after the menopause and characterized by persistent stiffness and pain in the joints affected." Slight crepitation of the

kaees was noted but limitation of motion or objective signs of joint disease were rare. Fisher<sup>18</sup> stated, "Disordered endocrine function plays an important part in many of the more chronic forms of arthritis occurring after the meridian of life. The type of arthritis that immediately springs to mind is the chronic osteoarthritis often seen in the knees of women, the onset of which corresponds with the menopause." The *Primer on Rheumatism*<sup>11</sup> stated that the menopause seems to be an exciting cause of hypertrophic arthritis. Jones<sup>19</sup> thought that osteoarthritis of the knees was most commonly met with in women at the menopause. Thomson and Gordou<sup>20</sup> thought climacteric arthritis was essentially the same as chronic synovitis which may eventually develop into typical osteoarthritis. Fox and Van Breemen<sup>1</sup> recognized menopausal arthritis as a generally accepted clinical entity. Radiographs show no change in bone and cartilage during the incipient stage which may be very long. Later the appearance resembles that of osteoarthritis. Steinbrocker<sup>2</sup> states that so called menopausal arthritis consists merely of arthralgia associated with vasospastic symptoms of ovarian hormonal deficiency and that the symptoms are not of articular origin. Rheumatic pain was found to occur as a symptom of the climacteric in 24 per cent of 1,197 women by the British Sub-committee of the Council of the Medical Women's Federation.<sup>23</sup>

#### DISCUSSION

The nature of the phenomena considered in this study is such that exact data are not always obtainable. The development of Heberden's nodes is a slow process and its beginning cannot be identified with certainty. The degree of development necessary for recognition depends upon the discernment of the patient as influenced by her temperament, her degree of introspection and her experience in having seen the deformity in her mother, sister, or intimate friends. Identification of the date of onset always seems more definite and apparently more exact the more recent it has been. It is almost equally convincing to hear that in one instance both events occurred about twenty years previously the fingers not enlarging until one year after the last menstrual period. An uncertainty is suggested by a statement that the last menstrual period was six years previously and that the fingers began to enlarge about twenty years ago. To discard such data because they seem to exact introduces a different and more significant error in the study by eliminating those cases in which onset and menopause were not closely associated in point of time. The mere fact that onset and menopause were separated by many years and that both events were remote casts doubt only upon the exact time relationship of the two events and not on the fundamental fact that their occurrence was separated by a wide lapse of time. In several instances patients were observed personally for years while these events were occurring. In Case 7 the patient continued to menstruate for six years after finger enlargement had first been noted. Her mother, Case 96, was seen before her fingers enlarged. A series of photographs of her hand made for another purpose showed the onset of nodes at about 62 years of age although her menopause occurred at 48 years. Cases were excluded only when the patients were definitely uncertain of the dates of the events under discussion.

Although the date of the last menstrual period was ascertained with reasonable certainty, the date of the onset or the duration of the climacteric was not easily identified. Removal of the uterus may stop menses without interfering with ovarian function. In Case 5 the uterus was removed when the

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# THE STATE OF COMPONENT A (PROTHROMBIN) IN HUMAN BLOOD, EVIDENCE THAT IT IS PARTLY FREE AND PARTLY IN AN INACTIVE OR PRECURSOR FORM

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THE simple concept expressed in the classical theory of Morawitz and of Fuld and Spiro that prothrombin is converted by thromboplastin (thrombokinas) and ionized calcium to thrombin has become untenable because of a series of recent findings. In 1943 Quick,<sup>1</sup> and the following year Owien,<sup>2</sup> discovered a new factor which is essential for the formation of thrombin. Recently many other investigators have contributed data to confirm and support these original observations, but as is to be expected in the development of a new subject numerous conflicting interpretations have been offered. No successful attempt has been made to correlate the pertinent contributions, particularly of Munro and Munro,<sup>3, 4, 5</sup> Fantl and Nance,<sup>6, 7, 8, 9</sup> Seegers, Ware and associates,<sup>10, 11</sup> and Alexander and co-workers.<sup>12, 13, 14</sup> Recently we have obtained experimental findings which appear to bridge the conflicting and seemingly hostile views on prothrombin conversion currently expressed.

In the study made in 1943<sup>1</sup> it was observed that when stored oxalated human plasma was mixed with an equal volume of dog plasma in which prothrombin had been reduced by Dicumarol or adsorbed with aluminum hydroxide the resulting mixture had a prothrombin time of 9 to 10 seconds, which was shorter than that of fresh plasma. Three years later,<sup>2</sup> it was found that human citrated plasma (1 vol of 0.1M sodium citrate to 9 vol of blood) often showed a reduction of the normal prothrombin time of 12 seconds to as low as 9 seconds after twenty-four hours of storage. No explanation for these findings could be offered until 1948<sup>15</sup> when it was recognized that two changes were concurrently taking place in stored plasma: namely a decrease in the labile factor and an increase in some other factor which augmented prothrombin activity. Due particularly to the development of an assay method for component A,<sup>4</sup> and also to the discovery of the effect of silicone-coated containers on prothrombin activity during storage, it has been possible to obtain data which offer not only an explanation for the increased prothrombin activity of aged plasma but also a new concept concerning the state of prothrombin in blood.

Since the terminology of the prothrombin factors has become increasingly more complex, it is essential that the principal terms employed in this study be defined. The labile factor is a constituent of both plasma and serum. It loses its activity when heated to 58° C and is diminished in stored plasma because of oxidation. Removal of ionized calcium increases the speed of inactivation. Little if any of the labile factor is adsorbed by the amount of tricalcium

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phosphate which completely removes component A. It is not reduced either in Dicumarol poisoning or in vitamin K deficiency. Rabbit blood contains approximately fifty times as much labile factor as human plasma.<sup>23</sup>

Component A of prothrombin probably corresponds to the classical prothrombin. It is inactivated at 58° C, but does not diminish during storage. It is completely adsorbed by tricalcium phosphate and can be quantitatively recovered by elution with sodium citrate. Component A is diminished in Dicumarol poisoning and in avitaminosis K. Evidence has been presented to show that this component combines with the citrate ion, thereby losing activity and becoming no longer adsorbable by tricalcium phosphate.<sup>25</sup>

#### METHODS AND MATERIAL

The reagents and procedures employed were essentially the same as those described in a recent paper.<sup>24</sup> Silicone coated glassware was prepared by means of Dri Film 9987 (General Electric), using the technique described by Jaques and associates.<sup>26</sup> For high speed centrifugation, an angle centrifuge which had a maximum revolutions per minute of 4,000 was employed. For slow speed the revolutions per minute were 800 to 1,000 in an ordinary centrifuge.

The method of isolating component A by adsorption with tricalcium phosphate and elution with sodium citrate, which we recently described,<sup>24</sup> concentrates the active factor ten times. In its assay, therefore, 1 vol was diluted with 9 vol of calcium phosphate treated plasma. Rabbit plasma was used because it has an exceedingly high concentration of labile factor. The concentrate of component A is called eluate in this paper.

#### RESULTS

*The Effect of Varying the Concentration of the Labile Factor on the Prothrombin Time When Component A is Kept Constant*—In a recent paper<sup>24</sup> it was shown that component A can be completely removed from oxalated plasma by an amount of tricalcium phosphate which takes out no detectable quantity of the labile factor. It was further shown that component A can be almost quantitatively recovered by elution with sodium citrate, and that this eluate when added to calcium phosphate plasma restores prothrombin activity provided sufficient labile factor is present. Since rabbit plasma contains fifty times and dog plasma ten times more labile factor than fresh normal human plasma and stored human plasma contains only a very small amount, treatment of these plasmas with tricalcium phosphate will yield testing media containing a wide range of concentrations of the labile factor. Such a series of deprothrombinized plasmas were employed to test the eluates from dog and human plasma. In the experiment, recorded in Fig 1, 1 vol of eluate was mixed with 9 vol of deprothrombinized plasma. It will be observed that when the labile factor was low the prothrombin time was prolonged, and as the labile factor was increased, the prothrombin time decreased and reached a fixed minimal value. Dog eluate which contains a higher concentration of component A required more labile factor to attain a fixed minimal prothrombin time. If the labile factor were acting catalytically, its activity should continue as its concentration increases.

These results therefore suggest that the reaction of the labile factor and component A is stoichiometric. We have further found (unpublished data) that

when plasma deficient in labile factor clots, very little component A is consumed even when thromboplastin is in excess and the calcium concentration is optimal, but as the labile factor is progressively increased, the utilization of component A rises in proportion. Since these results support the view that the reaction between thromboplastin, component A, and labile factor is stoecho

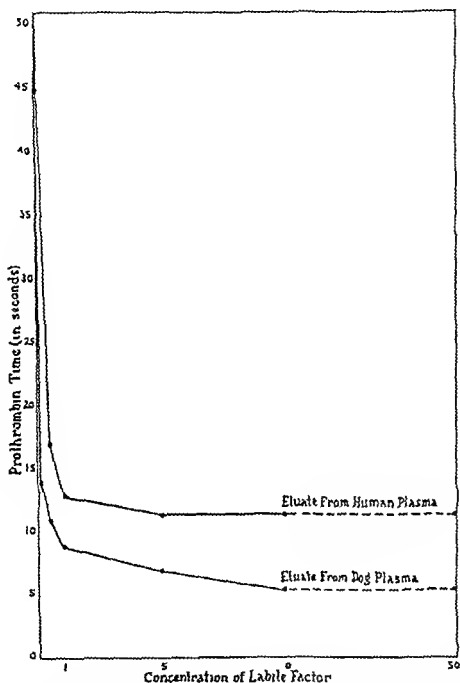
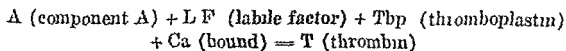


Fig 1—The influence of the concentration of the labile factor on the prothrombin time. The concentration designated as 60 was obtained by treating fresh oxalated rabbit plasma with tricalcium phosphate. 10 was dog plasma, a mixture of dog and stored human plasma, and 1 fresh human plasma. One volume of eluate was mixed with 9 volumes of the plasma treated with tricalcium phosphate.

metre, one should question the correctness of calling the latter an accelerator. It is interesting to note that when Ware, Guest, and Seegers<sup>11</sup> corrected the two stage method by adding an optimum amount of labile factor, they increased their yield of thrombin over 50 per cent. It can be postulated that at least three agents interact to form thrombin and that this reaction follows the law of mass action. The equation can be expressed as follows:



At present there is insufficient data to determine whether all three agents interact simultaneously or whether two react to form a product which enters into a reaction with the third to form thrombin.

*Evidence That Component A in Circulating Blood Exists Partly Free or Active and Partly in an Inactive State*—As already stated, when oxalated human plasma is stored in glass its prothrombin activity is markedly increased. Due to the fact that the labile factor is diminished on storage, this augmentation in prothrombin potency is masked and becomes demonstrable only by the addition of the labile factor. Interestingly, this increase in prothrombin activity does not occur or is greatly delayed if the plasma is stored in a silicone coated container. It should be mentioned that Alexander and associates<sup>21</sup> observed that when blood clotted in silicone apparatus, the prothrombin activity of the serum was abnormally high, and the factor which they call serum prothrombin conversion accelerator was decreased.

The pertinent facts are brought out by the following experiment. Blood was obtained from a normal subject. A silicone-coated syringe chilled in an ice bath was employed. The blood was immediately oxalated, transferred to cold silicone-coated tubes, and centrifuged. Part of the plasma was put in Pyrex test tubes and the remainder in silicone-coated tubes. The amount of plasma in each tube was the same, and all were stored in an ordinary refrigerator. The results are summarized in Table I.

TABLE I THE EFFECT OF TWENTY FOUR HOUR STORAGE OF OXALATED HUMAN PLASMA IN GLASS AND SILICONE COATED CONTAINERS ON COMPONENT A OF PROTHROMBIN

Rabbit $\text{Ca}_3(\text{PO}_4)_2$ plasma (cc)	0 00	0 01	0 02	0 03	0 04	0 05	0 06	0 07	0 08	0 09
Normal oxalated human plasma (cc)	0 10	0 09	0 08	0 07	0 06	0 05	0 04	0 03	0 02	0 01
Prothrombin Time (Sec)										
Fresh human plasma in sil cone	12½	12	12	12	12	12½	13½	15	20	25
Stored human plasma in sil cone	17	12½	12	12	12	13	14	16	19	26
Stored human plasma in glass	13	9	8	8	8	8½	9	10	12	15
Rabbit $\text{Ca}_3(\text{PO}_4)_2$ plasma						0 09 cc				
Eluate of oxalated human plasma						0 01 cc				
Prothrombin Time										
Fresh plasma eluate in silicone						12 sec *				
Stored plasma eluate in silicone						12 sec				
Stored plasma eluate in glass						7 sec				

\*The eluate transferred to glass and stored for twenty four hours gave a value of 7 seconds.

It is clear that the prothrombin time of the fresh plasma (12 seconds) was normal and that it was not increased by the addition of calcium phosphate treated rabbit plasma which is rich in labile factor. An eluate prepared from this plasma when added to rabbit calcium phosphate plasma yielded a prothrombin time of 13 seconds, which approximated the expected result. After twenty-four hours, the plasma in silicone-coated tubes lost labile factor, for the prothrombin time increased to 17 seconds, but component A remained unchanged since on adding rabbit calcium phosphate plasma a normal prothrombin time of



12 seconds was obtained. The eluate of this plasma yielded a prothrombin time of 12 seconds. In marked contrast, the plasma stored in glass, which had a prothrombin time of 13 seconds, showed when mixed with rabbit calcium phosphate plasma, i.e., with excess labile factor, a prothrombin time of 8 seconds. The eluate from this plasma yielded a prothrombin time of 7 seconds. Significantly, the eluate stored twenty four hours in glass likewise showed the same increase in prothrombin activity. Usually maximum increase in prothrombin activity of plasma stored in glass is attained in twenty four hours.

These results can best be explained by postulating that component A exists in a free form which is responsible for the prothrombin time of fresh plasma, and in an inactive state which becomes activated by storage. This activation requires a rough surface such as glass but neither calcium nor thrombin are essential. Preliminary studies indicate that this reaction is very likely also independent of platelets. Eventually the activation occurs also in silicone coated containers. No studies so far have been made to determine in what form inactive component A occurs i.e. whether it is combined or merely existing as an inactive precursor. Tricalcium phosphate adsorbs both forms which accounts for the fact that the eluate on storage increases in potency. The nature of the activation remains undetermined.

*The Concentration of Active and Inactive Component A in Hemophilic Blood*—In the development of the prothrombin consumption test,<sup>2</sup> it was frequently observed that the prothrombin time of serum was considerably shorter than the prothrombin time of plasma which in hemophilia as in healthy subjects is 12 seconds. The solution to this anomalous finding was obtained by a simple experiment. Blood was collected from a hemophilic subject whose Lee White coagulation time was 60 minutes. The usual silicone technique was employed. Part of the plasma was obtained by high centrifugation the remainder by slow centrifugation. The native plasma in aliquotes of 3 c.c. was transferred to glass and silicone coated test tubes. The prothrombin times were determined at regular intervals.

It will be observed from the results in Table II that the prothrombin time became progressively shorter during the course of an hour in the glass tubes but remained unchanged in the silicone tubes. At the end of forty five minutes, the prothrombin times of both the highly and slowly centrifuged plasmas were  $8\frac{1}{2}$  seconds. The eluate likewise which in the original plasma yielded a prothrombin time of 12 seconds decreased in one hour to 7 seconds for the two plasmas kept in glass test tubes. It is clear that hemophilic plasma contains a normal concentration of both free and inactive component A and that the activation of the latter occurs promptly in glass. The fact that the reaction occurs as fast in platelet poor as in platelet rich plasma makes it appear that the platelets play no direct part.

One of us (A. J. Q.)<sup>2</sup> has presented evidence showing that the hemophilic defect is a lack of thromboplastinogen, as a result of which the utilization of prothrombin is extremely small. Since hemophilic blood contains a normal concentration of both component A and the labile factor but only a trace of throm-

TABLE II THE CONCENTRATION OF FREE AND TOTAL COMPONENT A IN HEMOPHILIC BLOOD, THE ACTIVATING EFFECT OF GLASS SURFACE

TIME (MIN)	PROTHROMBIN TIME OF NATIVE HEMOPHILIC PLASMA (SEC)			
	HIGHLY CENTRIFUGED		SLOWLY CENTRIFUGED	
	GLASS (1) (SEC)	SILICONE (2) (SEC)	GLASS (3) (SEC)	SILICONE (4) (SEC)
0	11	11	11	11
10	11	11	11	11
20	10	11	10	11
30	9½	11	9	11
45	8½*	11	8½	11
120		11		11
Rabbit C <sub>12</sub> (PO <sub>4</sub> ) <sub>3</sub> plasma			0.09 cc	
Eluate of hemophilic plasma			0.01 cc	
Prothrombin Time				
Eluate of plasma 1		Before incubation	11 sec	
		After 60 min	7 sec	
Eluate of plasma 2		Before incubation	11 sec	
		After 120 min	11 sec	
Eluate of plasma 3		Before incubation	11 sec	
		After 60 min	7 sec	
Eluate of plasma 4		Before incubation	11 sec	
		After 120 min	7 sec	

\*Coagulation began

boplastin, nearly all of the prothrombin complex remains unconsumed after coagulation. Therefore almost all of component A remains even though hemophilic blood clots, and in addition the inactive A becomes converted to the free state with the result that total free or active component A greatly exceeds the amount initially present at the time the blood was drawn.

*The Effect of Removing Platelets on the Conversion of Inactive Component A*—It was early observed that the prothrombin consumption time in thrombocytopenia was, as in hemophilia, actually shorter than the prothrombin time of normal plasma.<sup>22</sup> The problem could now be studied more effectively. Blood was drawn in a chilled silicone syringe and transferred to silicone-coated test tubes immersed in ice. The tubes were centrifuged for thirty minutes at 4,000 r.p.m. in an angle centrifuge. Three cubic centimeters of the platelet-poor plasma were put in a silicone-coated test tube and an equal amount in a glass tube.

It will be observed from the results in Table III that the prothrombin time in the silicone tube remained unchanged during one hour, whereas it shortened to 8½ seconds in less than twenty minutes in the glass tube. An eluate prepared immediately from the native plasma gave a prothrombin time of 12 seconds, and a similar value was obtained from the plasma remaining one hour in a silicone-coated tube. The eluate from the plasma in the glass test tube yielded a prothrombin time of 8 seconds.

In seeking the reason for these abnormally short prothrombin times of platelet-poor plasma in a glass container, the most satisfactory explanation is found in Quick's theory that thromboplastinogen is activated by a platelet enzyme. Since the platelets have been removed almost completely, only a trace of the activating enzyme is available and therefore only a minute amount of

TABLE III. THE EFFECT OF GLASS AND SILICONE SURFACES ON THE PROTHROMBIN ACTIVITY (CHANGE IN CONCENTRATION OF FREE COMPONENT A) OF PLATELET POOR NORMAL NATIVE HUMAN PLASMA

PROTHROMBIN TIME OF NATIVE HUMAN PLASMA		
TIME (MIN)	GLASS (1) (SEC)	SILICONE (2) (SEC)
0	12	12
10	10	11½
20	3½*	11½
30		11½
60		11½
Eluate Tested by Mixing With 9 Parts of Rabbit Ca (PO), Plasma		
Plasma 1	Before incubation	12 sec
	After 20 min	8 sec
Plasma 2	Before incubation	12 sec
	After 60 min	12 sec

Coagulation began at 1½ minutes

thromboplastin is formed. As in hemophilia only a small fraction of the labile factor and component A is consumed in the reaction with thromboplastin, and since additional free component A is derived from inactive component A, the total amount of the latter is more than twice that of the original plasma.

TABLE IV. THE EFFECT OF SURFACE (GLASS AND SILICONE) ON THE PROTHROMBIN CONSUMPTION TIME WHEN THROMBIN\* IS ADDED TO PLATELET POOR NATIVE HUMAN PLASMA

TIME OF INCUBATION (MIN)	PROTHROMBIN CONSUMPTION TIME†	
	IN GLASS (SEC)	IN SILICONE (SEC)
0	12	12
15	9	11
30	8	11
75	8	11

\*To 1 cc of native plasma 0.01 cc of thrombin (Quick's full strength) was added.

†The prothrombin consumption test time was determined by the procedure previously described.\*

In order to determine whether thrombin is the agent responsible for the conversion of inactive component A to the active form native platelet poor plasma was placed in silicone and in glass test tubes and thrombin added to both. As seen from the results in Table IV there is no indication that thrombin caused any activation of inert component A in silicone. Whether it participated in the reaction occurring in glass is difficult to decide.

*The Effect of Dicumarol on the Concentration of Free and Inactive Component A*—Only preliminary studies have been made. The findings indicate that both free and the inactive component A are reduced. Further studies are in progress.

*The Relative Ratios of Free to Inactive Component A in Congenital Hypoprothrombinemias*—There are three known types of congenital hypoprothrombinemia.<sup>28, 29, 30</sup> In one the labile factor is diminished while in the other two this agent is normal but component A is diminished. In the first type according to Owien<sup>30</sup> who has reported the only case so far known in which the labile factor (Factor V) was apparently diminished from both the conventional prothrombin is quantitatively normal.

In types II and III of congenital hypoprotrombinemia the labile factor is normal. Both types show a prolonged prothrombin time by the one stage method, but curiously when 1 vol of type II is added to an equal volume of type III, the resulting mixture has a prothrombin time which is nearly that of normal plasma.<sup>29</sup>

To explain these results, Quick<sup>28</sup> postulated that one type (II) lacked component A and that the second type (III) was deficient in a hypothetical component B, and that when the plasmas of the two types were mixed in equal volumes they mutually corrected each other. With the new evidence obtained in this study that only a part of the total component A is present in the active state, a better interpretation of these findings can be offered and means to differentiate types II and III can be formulated.

From the results in Table V, the basic difference between the two types of congenital hypoprotrombinemia becomes clearly evident. In type II there is a definite lack of both free and total component A, whereas in type III, the free is diminished but the total may be practically the same as that of normal plasma. When plasma of type II is mixed with type III, sufficient inactive component A of the latter plasma is converted to the active form to bring the prothrombin time nearly to normal.

TABLE V THE CONCENTRATION OF FREE AND TOTAL COMPONENT A IN THE TWO TYPES OF CONGENITAL HYPOPROTHROMBINEMIA IN WHICH THE LABILE FACTOR IS NORMAL

Rabbit $\text{Ca}_2(\text{PO}_4)_2$ plasma (cc)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Congenital hypoprotrombinemia plasma (cc)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Prothrombin Time (Sec)										
Type II, fresh plasma	20	19½	19½	20½	21	22	23	26	35	53
Type II, after 4 days of storage in glass	37	17½	17	16	16½	16½	18½	21	28	42
Type III, fresh plasma	16	15	14½	15	15	16	17½	18½	22½	30
Type III, after 5 days of storage in glass	30	10	9	8	8	8½	8¾	9	11	17
The eluates were tested by adding 0.01 cc to 0.09 cc of rabbit plasma treated with $\text{Ca}_2(\text{PO}_4)_2$										
Prothrombin Time										
Eluate of fresh plasma, type II						17 sec				
Eluate of stored plasma, type II						16 sec				
Eluate of fresh plasma, type III						13 sec				
Eluate of stored plasma, type III						7 sec				

Apparently there is in plasma a regulating mechanism which determines the ratio of free to total component A. In normal human plasma, the ratio is such that a prothrombin time of 12 seconds is obtained with the one stage method. When all of component A is converted to the free state as occurs in storage, the prothrombin time becomes 7 to 8 seconds. Similar values are obtained when type III plasma is stored in glass even though the prothrombin time of the fresh plasma may be elevated significantly. Clearly, in this form of hypoprotrombinemia, the concentration of free component A is fixed at a level lower than normal. The fixation of the free prothrombin level appears definitely to be established by heredity. The affected members of the family who have

been studied in our laboratory<sup>8 29 31</sup> have a prothrombin time of 16 seconds. A second family has recently been studied in which the prothrombin time of the mother and her two children is 14 seconds. None of these subjects has a bleeding tendency. It is likely however that if the level of the free component A falls below a critical level a hemorrhagic diathesis will develop. The patient studied by Hagen and Watson<sup>32</sup> very probably has a type III hypoprothrombinemia, and she has a severe bleeding condition\*. In rabbits and dogs nearly all of component A is in the free state, according to our preliminary studies.

#### DISCUSSION

The findings reported in this paper promise to offer for the first time means to correlate and harmonize the significant contributions of Bordet,<sup>33 34</sup> Nolf<sup>35</sup> and the more recent investigators. It will be recalled that Bordet concluded that both cytozyme (thromboplastin) and serozyme (prothrombin) were mother substances of thrombin. He believed that the platelets liberated cytozyme when they came in contact with a rough surface. He further postulated that the serozyme in circulating blood was in an inactive state which he named proserozyme. For its activation, calcium and a rough surface were required. He concluded that tricalcium phosphate completely removed serozyme and assumed that the resulting 'phosphate plasma' contained only one remaining clotting factor, namely fibrinogen. Nolf<sup>35</sup> on the contrary, postulated that serozyme contained two factors, thrombozyme adsorbable by tricalcium phosphate and thrombogen which remained in the 'phosphate plasma'. Nolf unfortunately failed to recognize the importance of platelets and in his earlier work looked upon thrombin not as the cause of clotting but as a consequence of the reaction.

In agreement with Bordet, the present findings confirm the existence of a proserozyme but differ in that not all but only a part of the serozyme is in this inactive state. The activation of proserozyme i.e., the conversion of inactive component A, requires a rough surface like glass but apparently is not dependent upon calcium as Bordet assumed since the activation occurs in oxalated plasma. Bordet was not aware that phosphate plasma still contained a clotting factor other than fibrinogen. Undoubtedly the thrombogen of Nolf is identical with the labile factor.

In the development of this subject the establishment of the concept that the interaction of thromboplastin with the prothrombin complex is stoichiometric was of basic importance. Mertz Seegers and Smith<sup>36</sup> showed that the amount of thrombin obtained from a fixed amount of prothrombin increased proportionately to the quantity of thromboplastin added until the optimum was reached. Later Quick<sup>37</sup> showed that calcium likewise acted stoichiometrically in this reaction. With the discovery of the labile factor and the finding that it shortened the prothrombin time a new period of confusion set in. Quick postulated its action was stoichiometric whereas Owien<sup>38</sup> claimed that it functioned in forming a prothrombin converting enzyme. Fantl and Nance<sup>7</sup> who studied this factor concluded that it increased the speed of the conversion of prothrombin and they named it prothrombin accelerator. Munro and Munro

Dr. Paul S. Hagen kindly applied us with blood from this patient and it was found that she lacked both free and total prothrombin.

believed that prothrombin on storage became partly inactivated by an alteration of a labile group in the molecule and could again be reactivated, but they apparently did not consider that the activating agent was the labile factor. Seegers and associates, who are now inclined to agree<sup>18</sup> that the agent which they named Ac-globulin is the same as the labile factor, assumed that it increased the conversion rate of prothrombin. Murphy and Seegers<sup>17</sup> attempted to explain the discrepancies in prothrombin values of different species obtained by the one- and two-stage methods on the variations of the concentration of Ac-globulin.

As already mentioned, we have presented experimental findings which show that increasing the concentration of labile factor manifold did not decrease the prothrombin time<sup>24</sup>. Assuredly, the prothrombin time should be a sensitive test of the speed of conversion of prothrombin. In our studies, we learned that consistent results were obtainable only if silicone glassware was used. Since Bordet's work had been forgotten, it was generally assumed that a rough surface influenced only platelet disintegration, with the result that conversion of inactive component A to the active state which occurred was not recognized and was misinterpreted as a conversion of prothrombin to thrombin. The serum prothrombin conversion accelerator of Alexander and co-workers<sup>20</sup> is very probably concerned with the activation of component A rather than with the conversion of prothrombin to thrombin.

Many of the findings on Ac-globulin by Seegers and his associates are difficult to interpret because much of their material was collected in slaughter houses and generally in large containers. Under such conditions, considerable changes could occur even before the blood was decalcified, especially since sodium oxalate is relatively slow in its action<sup>24</sup>. Fahey, Ware, and Seegers<sup>18</sup> observed no difference in the influence of silicone and glass surfaces on the stability of Ac-globulin, which agrees with our observations. They failed to note however that a glass surface has a basic influence in converting inactive component A to the active state, because the methods of study which they employed did not distinguish between free and total prothrombin (component A).

A rational explanation for the differences in prothrombin determination by the one- and two-stage methods can now be offered. By the one-stage procedure, the prothrombin activity of dog blood is almost five times as great as that of human blood, whereas by the two-stage method, the prothrombin of dog blood is only slightly higher than in human blood. As already stated, the prothrombin time of fresh human plasma is 12 seconds, but the time of stored blood with the labile factor restored may be 7 to 8 seconds. The prothrombin time of dog plasma is 6 seconds. Since it can be assumed that in stored blood all the component A becomes activated, one can see that the prothrombin activity of dog plasma and of human plasma with all of its prothrombin activated is of the same order of magnitude. In carrying out the two stage method, the conditions are such that all of the prothrombin (component A) is converted to the active form. Therefore the method measures only total component A. The one-stage method, on the contrary, measures only free or active component A. The fact that component A can be quantitatively adsorbed and eluted and then tested on a standardized deprothrombinized medium makes it possible to

check the results of the one stage method. By this means it has been found that dog plasma contains over four times as much free component A as human plasma.<sup>4</sup>

The establishment that component A, or the classical prothrombin, exists in human blood both in the free and in the precursor state creates a new problem. How and when does the inactive prothrombin become available in physiologic coagulation? From test tube experiments it is clear that a rough surface is required, but it is still uncertain whether such a surface is available intracorporally. It still remains to be determined whether the precursor component A functions directly in coagulation or whether it serves as a supply of free component A.

It is obvious that the terminology now in general use is cumbersome and in some instances inaccurate. We have adhered to the term labile factor since it is descriptive and noncommittal. We see no justification for calling it an accelerator. Eventually when its action is better defined, a more suitable name may be found. In regard to component A it appears fairly certain that it is or is closely related to the classical prothrombin, the serozyme of Bordet, and the thrombozyme of Nolf. If component A be named prothrombin, it becomes difficult to decide what its inactive or precursor compound should be called. One term which suggests itself is prothrombinogen. Since such a term in recent enzyme chemistry implies that in its activation cleavage occurs, it seems advisable to postpone action until such time when the mechanism has been elucidated. For the present the neutral terms component A and pre A seem best.

#### SUMMARY

1 From studies based on the prothrombin time of plasma and from data obtained with the adsorption and elution of component A, it is concluded that the formation of thrombin requires component A, the labile factor, thromboplastin and combined calcium. This reaction appears to follow the law of mass action. There is no evidence that any of the factors are accelerators.

2 In fresh human plasma component A (prothrombin) is present partly free and partly in an inactive or precursor state which corresponds to the proserozyme of Bordet. It requires a rough surface for its activation, but there is no evidence that calcium, thromboplastin or thrombin are essential for its conversion to the active state. In ovalated human plasma nearly all of component A is changed to the active form after twenty-four hours of storage if kept in contact with a rough surface such as glass.

3 In the coagulation of plasma in which there is a deficiency of available thromboplastin due either to a lack of thromboplastinogen as in hemophilia or to removal of platelets, the concentration of free component A is markedly increased because little of the original active form is consumed and additional active component A is produced from the inactive precursor.

4 Two types of congenital hypoprothrombinemia are known in which a deficiency of component A occurs. In one type a true deficiency of both free and total component A exists. In the second type, which is the more common and is hereditary, the concentration of total component A is normal but the

amount of free or active is below normal. This suggests that a mechanism, still completely unknown, regulates the ratio of active to total component A.

5 The present work confirms the early conclusions of Boidet that serozyme or prothrombin may exist in an inactive or precursor state, but, unlike Boidet's concept that all exists as proserozyme, the present findings show that a large fraction of the total component A is in an active form. It appears probable that in a number of recent studies the activation of component A has been misinterpreted as an acceleration of the conversion of prothrombin to thrombin.

6 Since the procedure of the two-stage method for determining prothrombin is such that all of the component A is converted to the active form, this method determines total prothrombin (component A) but does not distinguish between the free and inactive form. The one-stage method, on the contrary, determines the free or active component A and, to determine the total, all of component A must first be converted to the active state.

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# FAILURE OF SENSITIZED SHEEP CELL AGGLUTINATION TO CLARIFY THE DIAGNOSIS OF RHEUMATIC DISEASE

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## INTRODUCTION

EARLY joint pain in a patient often presents a difficult diagnostic problem. For this reason, the report by Rose, Ragan, Pearce, and Lipman<sup>1</sup> of a test that was diagnostic for active rheumatoid arthritis was received with much enthusiasm. However, much more study is indicated before the reliability of this test is established and before it can be placed in the hand of the clinician as a proved aid to the diagnosis of active rheumatoid arthritis.

Rose and co-workers<sup>1</sup> found that the sera of patients with active rheumatoid arthritis agglutinated sheep cells sensitized with rabbit anti-sheep cell serum in a much higher titer than they agglutinated plain sheep cells. In their series, the sera of twenty-seven patients with active rheumatoid arthritis were tested. Four of the sera had differential titers of 16 and the remainder had differential titers exceeding this level. Also reported in this series were sixteen cases of inactive rheumatoid arthritis, five of which had differential titers exceeding 16, while the remainder had differential titers ranging from zero to 16. Included in their report were twenty-nine patients without evidence of arthritis. Two of these patients had a differential titer of 16, while the remaining twenty-seven had a titer of eight or less.

Cases of rheumatoid spondylitis, rheumatic fever, arthritis other than rheumatoid arthritis and rheumatic fever, infectious mononucleosis, and Still's disease were included in their study, all but two demonstrating differential agglutination titers below 16. They concluded that a sixteen fold difference or more occurs almost exclusively with the sera of patients with rheumatoid arthritis, and that the magnitude of the difference in titer usually reflects the clinical activity of the disease.

It is the purpose of this paper to present our results with this procedure, to correlate, if possible, the results of this test with the activity of the disease, and to determine how much promise it might hold for the clinician in the differential diagnosis of the arthritides.

## METHODS AND MATERIALS

In our initial series we used the method of Rose and associates<sup>1</sup> as follows. The serum of patients with active rheumatoid arthritis was diluted serially and added to 1 per cent suspension of normal sheep erythrocytes in one set of tubes, and in another set of tubes the same serum in the same dilutions was added to a similar amount of 1 per cent suspension of sensitized sheep erythrocytes. Rose and co-workers<sup>1</sup> report agglutination up to 1+ occurs with the sensitized sheep erythrocytes in a serum dilution that is sixteen times (or more) the

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dilution with the normal sheep erythrocytes. Sheep erythrocytes were sensitized by 2 units of rabbit anti-sheep cell serum prepared by the technique used for the Kolmer complement fixation test.<sup>2</sup> The normal sheep erythrocytes were prepared from fresh defibrinated blood by washing the cells three times in saline (0.85 per cent) centrifuging, and resuspending in saline to make a 1 per cent suspension by volume.

Dilutions of rabbit anti-sheep cell sera ranging from 1:1000 to 1:10000 were made in saline, 0.5 ml of each dilution was then mixed with 0.5 ml of a 2 per cent suspension of washed sheep cells and to each tube 0.1 ml of a 1:10 dilution of fresh guinea pig complement was added. The tubes were incubated in a water bath at 37° C for one hour and the highest dilution of the serum producing complete hemolysis was considered to contain 1 unit of hemolysin.

When doing the agglutination test the rabbit anti-sheep cell serum was diluted so as to contain 2 units of hemolysin and mixed with an equal volume of a 2 per cent suspension of sheep cells.

This method determines the unit of sensitizing antibody according to its hemolytic titer, but, as will be discussed later, we were unable to obtain differential titers exceeding +8 by this technique. Rose<sup>3</sup> later suggested the agglutinative titer of the serum rather than the hemolytic titer as a more reliable index. Therefore the greater number of our determinations were made with the agglutinative titer.

For the agglutinative titer, dilutions of rabbit anti-sheep cell serum from 1:100 up, in steps of 100 in a volume of 0.25 ml were set up, to each tube 0.25 ml of a 2 per cent suspension of sheep erythrocytes was added. The tubes were incubated at 37° C for one hour and then kept in the icebox overnight. After centrifugation in order to obtain more definite agglutination, readings were taken. The highest serum dilution giving visible agglutination was taken as the agglutinative titer. For example if the titer were 1:500, a dilution of 1:1000 was used.

Whether the hemolytic or agglutinative titer was used the remainder of the test was set up identically. Serum was obtained from freshly collected venous blood of patients and the complement was inactivated by heating at 50° C for thirty minutes. Dilutions of serum in duplicate (twelve tubes each) containing 0.25 ml saline were made. To each of one series was added 0.25 ml of 1 per cent suspension of normal sheep erythrocytes and to the other series 0.25 ml of the 1 per cent suspension of sensitized sheep erythrocytes. The final serum dilutions were therefore 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, 1:8192. These were mixed well and placed in the icebox and read in twenty-four hours. In reading the results the highest dilution of serum showing a 1+ agglutination was recorded as the end point. The differential titer was the highest dilution of the serum agglutinating sensitized sheep erythrocytes to 1+ divided by the highest dilution of the serum agglutinating normal sheep cells to 1+. For example, if sensitized sheep cells were agglutinated to 1+ in a serum dilution of 1:128 and the normal sheep cells agglutinated to 1:10 then the differential titer would be 8. (This can be referred to as the "algebraic difference".)

In obtaining material for this study it was decided to include all cases of arthralgia and cases of the so-called collagen diseases. But after examining twelve patients it was apparent that the differential titers being obtained were not high enough to be significant. At this time the technique was reviewed and the decision made to use the agglutinative titer. Thereafter cases of rheumatoid arthritis, osteoarthritis, rheumatoid spondylitis, psoriatic arthritis, rheumatic fever, lupus erythematosus disseminatus and controls were used, but the primary interest was in active rheumatoid arthritis so that the reproducibility of the test could be determined.

## RESULTS

Using the hemolytic titer agglutination tests were performed with the sera of twelve patients. The results are tabulated in Table I. The differential titer never exceeded an eightfold difference even though this includes four cases of active rheumatoid arthritis. There was no correlation between activity and the differential titer.

TABLE I DIFFERENTIAL SHEEP CELL AGGLUTINATION USING THE HEMOLYTIC TITER

PA TIENT	DIAGNOSIS	SEX	AGE (YR)	SED RATE	WBC	NSC	SSC	DIFF TITER
E K.	Rheumatoid arthritis ac tive, with scleroderma and calcinosis	F	64	23	8,800	1 4	1 8	2
J H	Rheumatoid arthritis ac tive, osteoarthritis	F	65	25	8,800	1 4	1 4	0
R H	Rheumatoid arthritis ac tive	F	54	23	8,500	1 16	1 16	0
J S	Rheumatoid arthritis ac tive	M	54	24	9,100	1 16	1 128	8
M M	Rheumatoid arthritis in active	M	75	9	7,400	0	0	0
M S	Lupus erythematosus disseminatus	F	20	24	800	1 16	1 32	2
E S	Palindromic rheumatism	F	40	13	9,000	1 4	1 8	2
R M	Acute rheumatic fever, pregnancy (2 mo)	F	24	26	14,400	0	1 4	4
L K	Undiagnosed joint pain	M	29	25	14,800	1 4	1 16	4
B B	Bilateral sclerosing osteitis of sacrum	M	19	5	7,700	0	1 4	4
J F	Infectious mononucleosis	F	23	-	7,500	1 128	1 512	4
G L	Conversion hysteria with joint pain	F	24	7	5,800	1 8	1 16	2

NSC Normal sheep cells  
SSC Sensitized sheep cells

Using the agglutinative titer, agglutination tests were performed on the sera of forty-four patients and the results are summarized in Table II. The cases may be divided into the following groups: (1) active rheumatoid arthritis, (2) nonrheumatoid joint disease, (3) latent syphilis, and (4) controls.

The differential titers in the twenty-one cases of active rheumatoid arthritis ranged from 0.25, in which the normal sheep cells were agglutinated to 1.16 and the sensitized sheep cells to 1.4, to the highest differential of 64. These patients all had elevated sedimentation rates, joint pain, and objective evidence of joint disease. The magnitude of the difference in titer did not reflect the clinical activity of the disease nor was there any definite correlation between the differential titer and the erythrocyte sedimentation rate. It is interesting to note that one of the controls had a differential titer of 64. An average of the differential titers of the twenty-one cases of active rheumatoid arthritis is 21.56 which is above the sixteen-fold difference which has been designated by Rose and co-workers<sup>1</sup> as the upper limit of "normal." Table III summarizes these averages.

TABLE II DIFFERENTIAL AGGLUTINATION WITH AGGLUTINATIVE TITER—FORTY-FOUR PATIENTS

CLINICAL DIAGNOSIS	NUMBER OF CASES	DIFFERENTIAL AGGLUTINATION TITERS							
		0	2	4	8	16	32	64	128
Rheumatoid arthritis active	21	3		3	1	7	4	3	
Rheumatoid spondylitis	2				2				
Rheumatoid spondylitis with pe ripheral joint involvement	1				1				
Psoriatic arthritis	2	1				1			
Rheumatic fever—acute	3		1	2					
Lupus erythematosus disseminatus	1			1					
Osteoarthritis	1							1	
Infectious arthritis	1		1						
Syphilis	5				1	3	1		
Controls	7	2		3	1			1	

TABLE III. AVERAGES OF DIFFERENTIAL TITERS—FORTY FOUR PATIENTS

CLINICAL DIAGNOSIS	NUMBER OF CASES	DIFFERENTIAL TITER AVERAGES						
		0	2	4	8	16	32	64
Rheumatoid arthritis active	21	21 56						
Rheumatoid spondylitis	2	8						
Rheumatoid spondylitis with peripheral joint involvement	1	8						
Psoriatic arthritis	2	8						
Rheumatic fever	3	(3 33)						
Lupus erythematosus disseminatus	1	4						
Osteoarthritis	1	64						
Infectious arthritis	1	2						
Syphilis	5	17 6						
Controls	7	1						

The cases of nonrheumatoid joint disease included psoriatic arthritis two patients, rheumatoid spondylitis three patients one of whom had peripheral joint involvement, osteoarthritis one patient, active rheumatic fever, three patients, infectious arthritis, subsiding after evacuation of an ischiorectal abscess, one patient, and one patient with lupus erythematosus disseminatus. The patient with osteoarthritis had a differential titer of 64. One of the patients with psoriatic arthritis had a differential titer of 16. None of the others had differential titers exceeding 8. The average of the differential titers of this group was 10.9.

Another point of interest is the consistently high differential titer which was obtained with the sera of patients with latent syphilis but without subjective or objective evidence of joint disease. Five cases averaged a differential titer of 17.6 (see Table III) with differentials ranging from eightfold to thirty-two fold.

The seven control patients with no evidence of joint disease and with negative serologies included two cases of psychoneurosis, two normal individuals, one case of pericarditis, etiology undetermined, single cases of heart failure and hypertension. These controls had differential titers ranging from zero to 64 and the average of the differential titers was 12. This indicates that the dilution of the rabbit anti-sheep cell serum used in our studies was of the proper quality and that its agglutimative titer was satisfactory. Certainly, if reproducible results are to be obtained, such an adequate agglutimative titer is necessary.

It is apparent from the results obtained in this study that sensitized sheep cell agglutination is not specific enough to be of value as a diagnostic aid for active rheumatoid arthritis. Of the twenty-one cases of active rheumatoid arthritis, 66 per cent showed a differential titer of 16 or less. If the upper limit of "normal" were reduced to a differential of 8, 33 per cent of these cases were still "negative." It would not be feasible to lower this dividing line to 8 since

# EVALUATION OF AN IN VITRO HEPARIN TOLERANCE TEST FOR THROMBOEMBOLIC DISEASE

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THE need for a laboratory test to predict thromboembolic disease has been recognized for some time. Waugh and Ruddick,<sup>1, 2</sup> working along these lines, measured the ability of whole blood to counteract the anticoagulant effects of heparin in vitro. The basis for this test was the physiologic antagonism between thromboplastin and heparin, the former substance was considered to be increased in the bloodstream in cases of accelerated coagulation. By using increasing amounts of heparin in a series of test tubes, they attempted a "controlled deceleration of the clotting process." In interpreting the Waugh-Ruddick test, any curve that falls below the accepted normal range would indicate accelerated coagulability. On the other hand, results above the normal range would point to delayed coagulability.

Silverman<sup>3</sup> modified the test in an attempt at increasing its accuracy and eliminating certain inherent mechanical difficulties. In our studies at The Bronx Hospital, the Silverman modification of the Waugh-Ruddick test was used.

## METHOD

Serial dilutions of heparin were prepared as follows:

A stock solution was made containing 0.6 cc of heparin (1,000 units per cubic centimeter) in 300 cc of normal saline. The actual dilutions were made from the stock solution.

	STOCK SOLUTIONS (cc)	NORMAL SALINE (cc)	HEPARIN (UNIT PER 0.1 cc)
1	0	50	0
2	5	45	1/50
3	10	40	2/50
4	15	35	3/50
5	20	30	4/50
6	25	25	5/50
7	30	20	6/50
8	35	15	7/50

Four and one half cubic centimeters of blood were drawn and added to a tube containing 0.5 cc of 0.1M sodium oxalate. This was centrifuged to separate the plasma, 0.1 cc of plasma was placed in each of eight tubes (100 by 13 mm). To each tube 0.1 cc of one of the correspondingly numbered heparin solutions was added. Then 0.2 cc of 0.01M calcium chloride was added to each tube to offset the oxalate previously used and the time was noted. All tubes were then immediately placed in a water bath at 37.5° C and the rack was gently tilted periodically until coagulation was noted. Invariably the numbered tubes clotted in the expectedly orderly sequence.

The following precautions were taken to insure accuracy and uniformity of the test:

1. Stock solutions were kept at a temperature of 4° C when not in use.
2. The Wassermann type of tubes were of uniform size and scrupulously clean.
3. Venepuncture was done carefully and a clean dry syringe and needle were used.

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4 The test was always performed in a water bath of constant suitable temperature as suggested by Whittaker <sup>4</sup>

5 At periodic intervals the heparin solutions were checked for stability. Determinations were performed on the plasma of a normal individual using both old and freshly prepared solutions. At intervals of two to three weeks these results checked closely. Likewise, a new unopened vial of heparin (1,000 units per cubic centimeter) was checked against the old one and again a close correlation in results was noted.

6 The test was performed at about 10 A.M. in each instance.

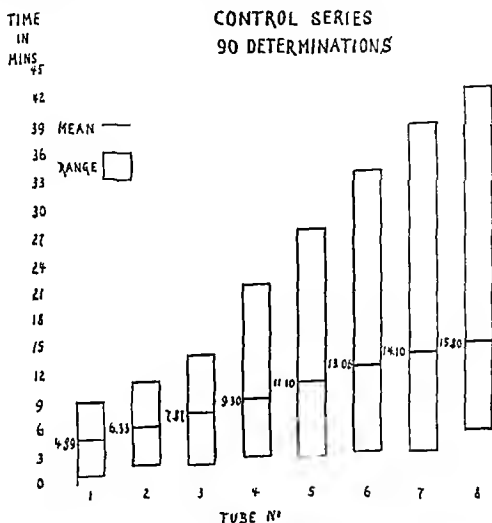


Fig 1—This graph shows the range and mean clotting times for each tube in ninety control determinations.

### RESULTS

Seventy four individuals were used as controls and ninety determinations were performed on this group. The controls varied in age from the third to the seventh decade and none showed clinical evidence of heart disease, thromboembolic phenomena, or blood dyscrasias. All were ambulatory. Our findings with this group are recorded in Fig 1. It will be noted that as the tubes contain increasing amounts of heparin the mean clotting time increases. The average increase in clotting time from tube to tube is 1.56 minutes with an actual range of difference from 1.04 minutes to 1.80 minutes. Attention is directed to the wide scatter of clotting times among normal individuals, especially in the tubes containing larger amounts of heparin. Two and three determinations were performed on some of the controls to note the variation in heparin tolerance not only among different individuals but also in the same person from day to day.

A few individuals showed a close correlation on repeated determinations, while most showed marked variations. Fig 2 illustrates the different types of results obtained in two controls.

After having established the normal range, the test was performed on twelve obstetric patients and seven candidates for prostatectomy. In the obstetric cases a determination was made ante partum and forty-eight hours post partum,

### COMPARATIVE DATA IN 2 CONTROLS

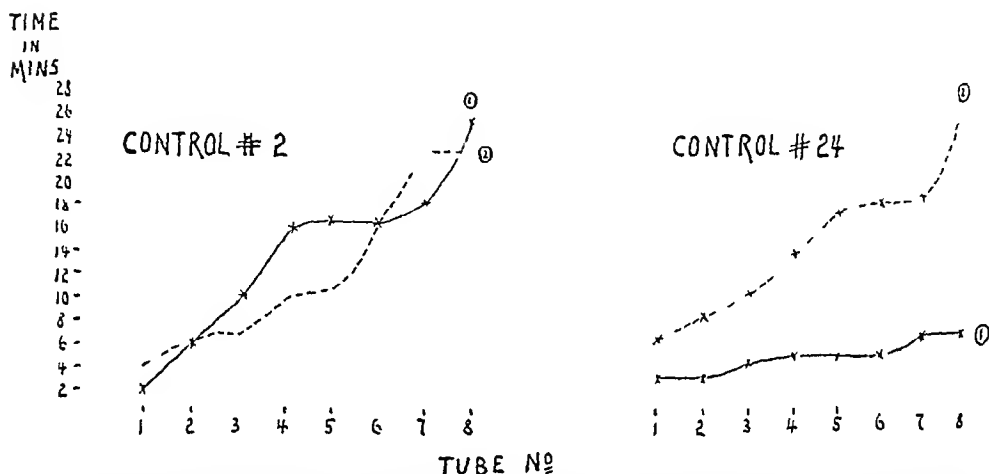


Fig 2—These curves illustrate the variation in results upon repeating the test in two controls on different days. In Control 2 the correlation is good while in Control 24 a marked difference in coagulability is noted. The solid line 1, in each control represents the first study; the interrupted line 2 is the repeat study.

and if an accelerated curve was then found a third test was performed seven days post partum. The same routine was followed in the prostatectomy cases. The initial determination in each case was included in our control series. Table I shows comparison of these groups and our controls, with a statistical analysis of the results. The difference between the standard deviations of the control group and the obstetric postoperative group is smaller than 3 sigma, in the case of each of the eight tubes. Thus, none of the tubes shows a significant statistical difference from the control to the obstetric-postoperative group.

TABLE I STATISTICAL ANALYSIS OF MEAN DIFFERENCES OF SERIAL CLOTTING TIMES BETWEEN CONTROL AND POSTOPERATIVE GROUPS

TUBE	CONTROLS (90)		POSTOPERATIVE PATIENTS (19)		ANALYSIS	
	MEAN CLOTTING TIME*	STANDARD DEVIATION	MEAN CLOTTING TIME	STANDARD DEVIATION	DIFFERENCES BETWEEN MEANS	TEST FOR SIGNIFICANCE (3σ)
1	4.87	1.77	4.04	1.56	0.83	1.23
2	6.33	2.01	5.44	1.80	0.89	1.35
3	7.87	2.74	6.59	2.46	1.28	1.86
4	9.30	3.72	7.74	2.79	1.56	2.19
5	11.10	5.10	8.85	4.05	2.25	3.21
6	13.06	6.21	9.85	4.50	3.21	3.63
7	14.10	7.20	10.63	5.40	3.47	4.26
8	15.80	8.76	11.44	6.18	4.36	5.64

\*All clotting times expressed in minutes



## DISCUSSION

In clinical application, preliminary studies by Waugh and Ruddick showed that an accelerated type of curve was obtained under the following circumstances

- 1 During uncomplicated bed rest
- 2 Following operative procedures
- 3 In the presence of acute infections
- 4 In the presence of hemorrhage

Prolongation of the curve was noted in patients undergoing radiation therapy

In performing his test on nine operative patients Silverman<sup>3</sup> noted a definite increase in coagulability following operation. This began within twenty-four hours after operation and lasted for approximately one week. Concomitant platelet counts were done and no increase was found until six or seven days after operation, so that thrombocytosis was not a significant factor in these results in the early postoperative period.

Further application of the principles of the Waugh-Ruddick test by Ogura and co-workers in twenty-seven cases of coronary thrombosis revealed an accelerated coagulation curve in 77.8 per cent of the cases. The onset and duration of this acceleration corresponded with the time range pointed out by Silverman as noted. A total of sixteen controls was used in this series, seven were normal individuals and nine were cardiac patients without thrombosis and at bed rest. Conventional tests for coagulation performed alongside this *in vitro* heparin tolerance test failed to detect any acceleration. The conclusion reached by these authors was that the accelerated coagulation did not cause the coronary thrombosis but was a result of myocardial destruction.

De Takats<sup>6</sup> made use of an *in vivo* heparin tolerance test as an aid in the diagnosis of thromboembolic disease. Ten milligrams of purified heparin were injected intravenously and coagulation times determined by the capillary tube method before the test and at ten minute intervals for forty minutes thereafter. He found a group of patients who were resistant to heparin. These were patients in the early postoperative period, patients who had suffered cardiovascular accidents, and patients with Buerger's disease.

The heparin tolerance curves in the post partum and postoperative states in our patients did not vary significantly from those obtained in the control series. While there may be a difference in the degree of coagulability of the blood of post partum and postoperative patients as compared with normal individuals, our results fail to demonstrate this. This may possibly be due to inherent inadequacy of the test.

## SUMMARY

An *in vitro* heparin tolerance test for the prediction of thromboembolic disease was studied in seventy-four controls, twelve obstetric patients and seven patients who had prostatectomies. The results were subjected to statistical analysis. In our hands this test failed to show a significant difference between the control and the post partum/postoperative groups. This differs from the

reports of previous investigators<sup>2 3</sup> In view of this discrepancy, further investigative work with this test and on other factors affecting blood coagulation seems indicated in order to clarify their place in the prediction of thromboembolic disease

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## OBSERVATIONS ON THE COAGULATION DEFECT IN THROMBOCYTOPENIC PURPURA

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THE presence of an excess of heparin or of a heparin like substance in the blood of patients with idiopathic or secondary thrombocytopenic purpura and other hemorrhagic states has been postulated by Allen and co workers<sup>1</sup>. This opinion was developed in part as the result of protamine heparin titrations and also by clinical observations on the effect of protamine and toluidine blue on abnormal bleeding. These agents are capable of reacting with several endogenous compounds in the body some of which are heparin like while others appear to be unrelated to heparin<sup>2</sup>. They were able to show that higher concentrations of protamine were necessary to cause coagulation of heparinized blood from patients with thrombocytopenic purpura than from normal persons. They also noted temporary cessation of hemorrhagic phenomena in some patients following intravenous administration of protamine or toluidine blue in a dosage of 1 to 4 mg per kilogram of body weight. Attention had been directed to this 'heparinoid state' by the hemorrhagic tendency which appeared in dogs following total body irradiation (roentgen ray)<sup>3,4</sup>. Allen and co workers found in the blood of these hemorrhagic animals a circulating anticoagulant which reacted in many ways similarly to heparin and the bleeding tendency could be controlled temporarily by the intravenous injection of protamine sulfate or toluidine blue.

Holmgren and Wilander<sup>5</sup> in 1937 found that acidic heparin was removed completely from blood in vitro by adding the 'basic' dye toluidine blue. This was apparently a precipitation phenomenon. Blood to which heparin is added has a prolonged coagulation time but the addition of a solution of toluidine blue will cause heparinized blood to clot in a few minutes. Chargoff and Olson<sup>6</sup> observed that the 'basic' amine, protamine, had an effect similar to that of toluidine blue but this action was apparently mediated through its effect on the electrical charge of heparin. Jorpes injected a protamine intravenously in man without untoward reaction and from in vitro studies concluded that protamine would neutralize heparin in the proportion of six parts of protamine to ten parts of heparin. Parkin and Kvale<sup>7</sup> concluded, from in vitro studies however that protamine neutralized heparin in a one to one ratio. There are several factors involved in blood coagulation through which heparin may act, but the exact mode of its action is not known. Heparin, when added in increasing amounts to normal blood, first shortens and then lengthens the whole blood

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clotting time<sup>9, 10, 11</sup> The amount of heparin necessary to shorten the clotting time has been shown to vary from 0.0001 to 0.01 unit per cubic centimeter of blood. One-tenth unit of heparin per cubic centimeter of blood will prolong the clotting time. This prolongation may be due to an effect on thrombin, prothrombin, thrombokinase, or possibly on other systems<sup>12, 13, 14, 15</sup>

The mechanism by which protamine alters the coagulation process has not been determined and it too may act in one of several different ways<sup>15, 19</sup>. An antithromboplastic or an antiprothrombic action in the first phase of blood coagulation has been postulated<sup>14, 15, 16</sup>. It also has been suggested that it may have a fibrinoplastic activity in the second phase. In sufficient concentration protamines also have been shown to precipitate fibrinogen and thereby may prolong blood coagulation when given in excess. Utilizing protamine and heparin in an attempt to determine changes in the coagulability of blood, as is done in the protamine titration test, introduces many unknown factors.

#### METHODS

While utilizing small amounts of heparin for prolonging the coagulation of blood by the method of Waugh and Ruddick,<sup>20, 21</sup> it was noted that some patients who were actively bleeding had an augmented reaction to heparin. The Waugh-Ruddick test was originally devised as a modification of the blood coagulation test of Lee and White<sup>22</sup> to provide a controlled deceleration of the coagulation process. The technique is as follows<sup>20, 21</sup>:

Nine Wassermann tubes (100 by 13 mm), thoroughly cleaned and dried, are placed in a rack. Tube number one remains empty until the blood is added. Tube number two contains  $\frac{1}{2}$  c.c. of  $\frac{9}{10}$  per cent normal saline. Serial dilutions of heparin in  $\frac{1}{2}$  c.c. of saline are added to the remaining seven tubes so that tube three receives 0.1 unit of heparin in  $\frac{1}{2}$  c.c. saline, tube four receives 0.2 unit of heparin in saline, and so on until tube nine receives 0.7 unit of heparin. To each of the tubes is added 1 c.c. of blood drawn with a minimum of trauma, using a number eighteen needle and measured from the same 10 c.c. syringe in which it was drawn. The tubes are stoppered and tipped to approximately 85 degrees at two minute intervals until coagulation occurs. The time which elapses from the moment blood is first seen in the syringe until coagulation occurs is the end point in that particular tube.

The test has been used to study the increased speed of coagulation of the blood of patients with myocardial infarction and other thrombotic conditions.<sup>23</sup> It has not been used heretofore, to our knowledge, in a study of patients with hemorrhagic tendencies.

#### RESULTS

The test was performed on thirty normal subjects and the results, as presented in Fig. 1, are in agreement with those of Waugh and Ruddick. The time of coagulation in tubes 1 and 2, which contain no heparin, varied from 4 to 12 minutes. In tube 3, containing 0.1 unit of heparin, coagulation occurred in from 12 to 40 minutes. A gradual increase in the time required for coagulation was found as increasing amounts of heparin are encountered. Coagulation ultimately occurred in from 40 to 90 minutes. In no instance was the blood rendered incoagulable under these conditions. The two extremes in the time of

coagulation in normal subjects are shown by the solid lines in Fig 1, while individual readings on each of the thirty patients are represented by the dots

When the Waugh Ruddick test was tried on the blood of patients who were actively bleeding because of thrombocytopenic purpura it was noted that there was an increased sensitivity to heparin as indicated by an increased time for coagulation in the tubes containing this material. Consequently the test was performed on fifteen patients who had bleeding tendencies associated with pri

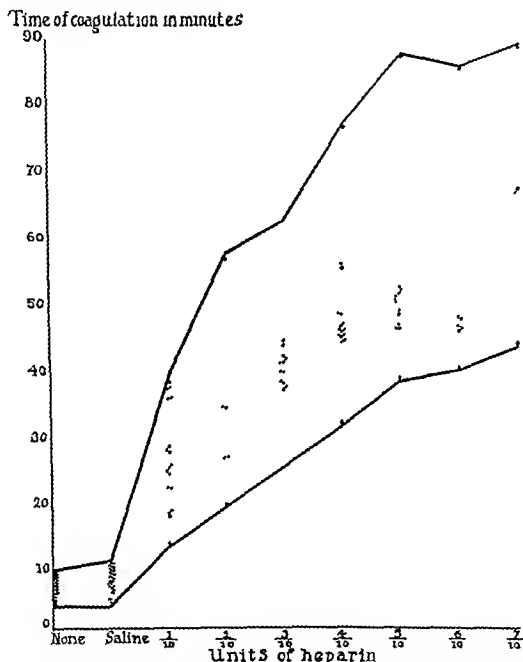


Fig 1—Showing the response to the Waugh Ruddick test in thirty normal individuals. The lines represent the two extremes.

mary or secondary thrombocytopenia. The patients and their diagnoses are listed in Table I, nine of them being classified as idiopathic thrombocytopenic purpura. The laboratory findings were typical in all cases: the clotting time was essentially normal, there was prolongation of the bleeding time, the thrombocytes were diminished, clot retractility was absent, and they presented clinical manifestations of hemorrhage. In three patients the thrombocytopenia was secondary to leucemia. It was secondary to a chemical agent in Case 13, and in Case 14 autopsy revealed a diffuse reticuloendotheliosis. Patient J. B., Case

TABLE I

CASE	PATIENT	CAUSE	PLATE LETS (THOU SANDS)	BLEEDING TIME (MIN)	CLOTTING TIME (MIN)	CLOT RE TRAC TILITY	HEMOR RHAGIC PHENOM ENON
1	D M	Idiopathic	8 0	10+	5 0	None	Yes
2	W M	Idiopathic	5 0	7	5 0	None	Yes
3	M S	Idiopathic	19 0	15+	4 0	None	Yes
4	J G	Idiopathic	4 0	10+	6 0	None	Yes
5	M P	Idiopathic	2 0	10+	5 0	None	Yes
6	J S	Idiopathic	5 0	10+	5 0	None	Yes
7	R P	Idiopathic	10 0	10+	5 5	None	Yes
8	S L L	Idiopathic	8 0	10+	6 0	None	Yes
9	L B	Idiopathic	12 0	10+	6 25	None	Yes
10	J R	Lymphatic leucemia	5 0	10+	8 0	None	Yes
11	F S	Myelogenous leu cemia	36 0	20+	6 0	None	Yes
12	W J W	Monocytic leucemia	2 0	10+	6 0	None	Yes
13	F R	? Toxic agent	4 0	10+	3 5	None	Yes
14	C D	Reticuloendothelial hyperplasia	5 0	10+	4 5	None	Yes
15	J B	Aplastic anemia	2 0	8 5	4 0	None	Yes

15, had aplastic anemia. One of the nine patients with idiopathic thrombocytopenic purpura died of cerebral hemorrhage before splenectomy could be performed. Splenectomy was done in eight patients, seven of whom responded satisfactorily with a return of the platelet count and bleeding time to normal and cessation of the hemorrhagic tendency. The eighth patient responded temporarily but reverted to the previous hemorrhagic state.

The studies on these patients with thrombocytopenic purpura revealed an essentially normal coagulation time in the first two tubes, which do not contain heparin, but a markedly prolonged coagulation time in the remaining tubes containing serial dilutions of heparin. Fluctuations were noted in the speed of coagulation in individual patients from time to time when there was no significant change in the number of platelets, but when there were active clinical hemorrhagic tendencies the increased sensitivity to heparin was always present. The variations in the Waugh-Ruddick test more nearly paralleled the clinical manifestations of hemorrhage than did the platelet level.

Fifty-six determinations were done on these fifteen patients and the results are presented in Fig 2. The solid line is a typical Waugh-Ruddick curve in a normal subject, presented for comparison. Coagulation occurred in 20 minutes in the presence of 0.1 unit of heparin, in 32 minutes with 0.2 unit, in 46 minutes with 0.3 unit, and in 84 minutes with 0.7 unit of heparin in the last tube. The shaded area in the figure represents the extremes in the response in fifty-six determinations on fifteen patients while they were actively bleeding. In these patients coagulation required from 45 to 100 minutes in the presence of 0.1 unit of heparin although the coagulation time in the first two tubes, containing no heparin, had been essentially normal. In the fourth tube, containing 0.2 unit of heparin, coagulation occurred in 60 minutes in one patient, but in many instances the blood was incoagulable in this dilution of heparin and in all subsequent dilutions. The shortest coagulation time with 0.3 unit of heparin was 80

minutes, but in most instances the blood was incoagulable with this dilution of heparin. There was a progressive lengthening of the coagulation time as the concentration of heparin was increased, and even in those having the shortest coagulation time it was much longer than the normal and an incoagulable state was reached in all cases. In none of the normal subjects was the blood made incoagulable by these dilutions of heparin. All determinations fell between the extremes represented by the shaded area. Incoagulability of the blood was usually produced with 0.3 to 0.4 unit of heparin and it remained incoagulable

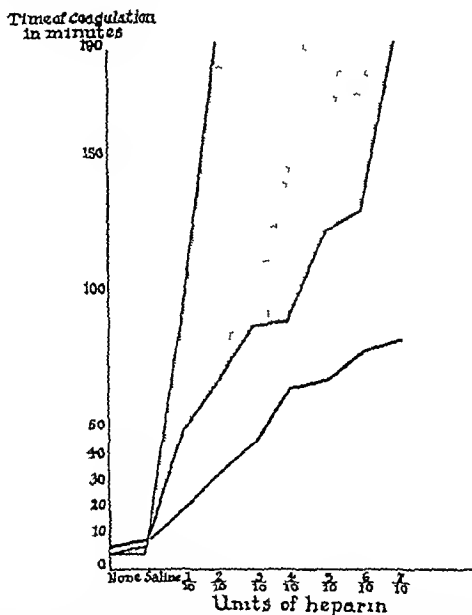


Fig. 1.—The heavy line shows the Waugh-Ruddick curve in a normal person. The shaded area represents the extremes in the response obtained in thrombocytopenic patients while actively bleeding. Blood from these patients is rendered incoagulable by this small amount of heparin. Although the chart is stopped at 190 minutes all blood samples were observed for twelve or more hours and were still incoagulable.

in subsequent tubes. These results show that the coagulation time in such patients is normal in the absence of heparin but is markedly increased with even small amounts of heparin and the blood is easily rendered incoagulable.

In four patients with purpura simplex (nonthrombocytopenic purpura) the Waugh-Ruddick test was normal.

Protamine sulfate and toluidine (1 to 4 mg. per kilogram of body weight) were administered to seven patients and then blood was studied in serial dilutions of heparin. Samples of blood were drawn prior to the administration of

protamine or toluidine blue, ten minutes after the administration was completed, and daily thereafter for variable periods. In two patients there was no demonstrable effect. In a third subject the Waugh-Ruddick curve returned to normal following the injection of protamine sulfate, but since the results persisted for more than one week it seems probable that the material was given concomitantly with a spontaneous remission. In the remaining instances the administration of protamine sulfate or toluidine blue apparently shortened the coagulation time in the tubes containing 0.1 and 0.2 unit of heparin and occasionally in the tube

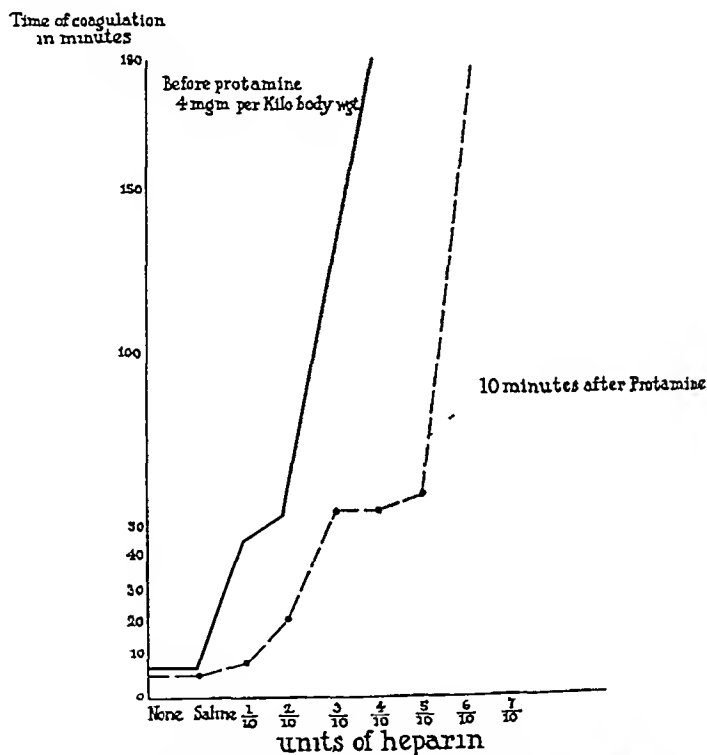


Fig 3—Typical Waugh-Ruddick curves on a thrombocytopenic patient before and after the administration of protamine sulfate. The blood did not clot and was observed for twelve hours although the chart is stopped at 190 minutes.

containing 0.3 unit of heparin but had no effect on the tubes containing a higher concentration. In these tubes the blood remained incoagulable. This suggests that the protamine in the blood stream was sufficient to neutralize 0.1 to 0.3 unit of heparin in tubes 3 and 4 but not larger amounts. Fig 3 shows a typical response in a patient before and after the administration of protamine. The partial flattening of the curve, apparently due to the presence of circulating protamine, was not noted in the twenty-four hour sample.

The Waugh-Ruddick test was done on five patients before and after splenectomy. In four of the cases the operation was followed by a complete clinical remission and a return of the platelet count and other laboratory findings to normal levels. In all of these patients the Waugh-Ruddick test also returned to normal. Fig 4 shows the results of this test in the four cases before and after

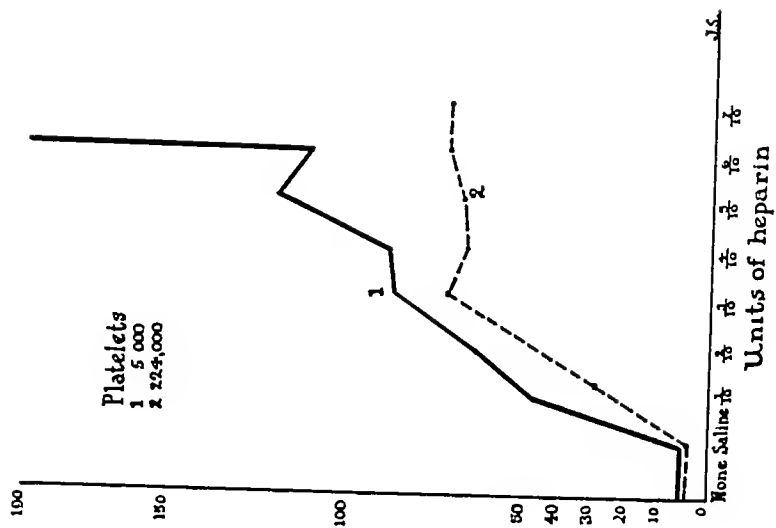
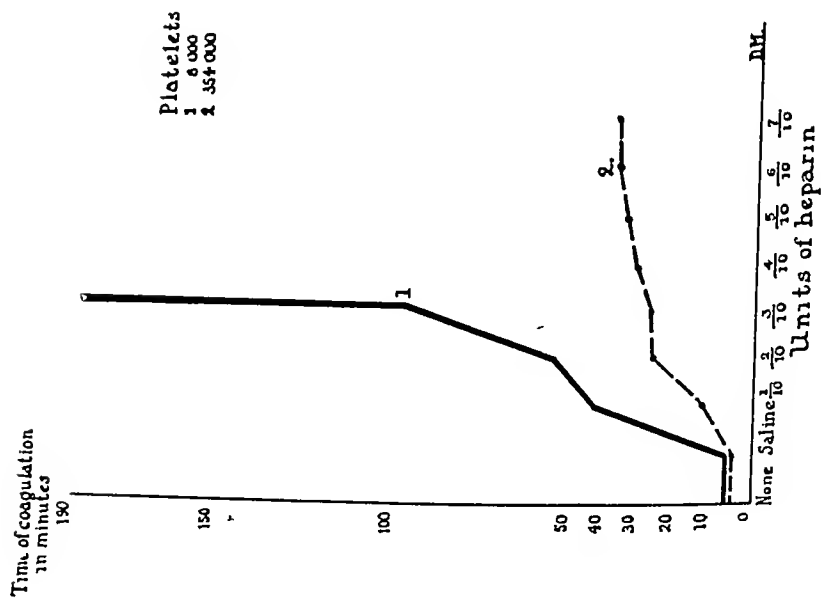


splenectomy. In the fifth case the operation was followed by an immediate but moderate rise in the platelet count and a normal Waugh Ruddick test. Two months later cutaneous hemorrhages had reappeared the platelet count had dropped to its previous low level and the Waugh Ruddick test was again even more prolonged than initially. Fig 5 shows the Waugh Ruddick test before operation, one hour postoperatively and two months later in the patient responding only temporarily to splenectomy.

#### DISCUSSION

These experiments show that the blood from patients with thrombocytopenic purpura exhibits an unusual sensitivity to heparin in that coagulation is extremely prolonged in its presence and the blood is rendered incoagulable when very small amounts of heparin are added. The prolongation of the coagulation time is less marked with normal blood and it does not reach an incoagulable stage under these conditions. This reaction is not like that produced by the intravenous injection of commercial heparin. The coagulation time of the whole blood as determined by the Lee White and capillary tube methods is not significantly prolonged in thrombocytopenic patients as is true when an excessive amount of commercial heparin is given. Toluene blue and protamine sulfate when given to these patients intravenously does not neutralize the incoagulability of the blood in the Waugh Ruddick procedure as would be expected if excess heparin were the basis for these changes. These substances appear only partially to neutralize the effect of the heparin used in the test. Further evidence against the presence of an excess of heparin in the blood of these patients was suggested by the administration of commercial heparin to normal subjects. The Waugh Ruddick curves in these individuals are not the same as curves obtained in thrombocytopenic patients. When heparin is given intravenously to a normal person in small amounts (10 mg) the coagulation time is only slightly prolonged and the blood does not become incoagulable in the Waugh Ruddick test containing the higher concentrations of heparin. When heparin is given in larger amounts (50 mg), the coagulation time is definitely prolonged but still the blood does not become incoagulable in the Waugh Ruddick test. This reaction differs from that of the blood of thrombocytopenic patients in that their blood always becomes incoagulable when tested during a period of hemorrhage and the Waugh Ruddick curve is entirely different from that of blood heparinized *in vivo*. These observations agree with the conclusion of Conley, Hartmann, and Morse<sup>25, 26</sup> that the increased susceptibility of thrombocytopenic blood to heparin apparently is not due to an increased amount of circulating heparin like substance.

The blood of patients with thrombocytopenic purpura is more sensitive to heparin *in vitro* than the blood of normal persons. This sensitivity closely parallels the clinical manifestations of hemorrhage and follows the clinical state more closely than it does the platelet count. The parallelism between the clinical manifestations of hemorrhage and heparin sensitivity is also closer than that between the clinical manifestations and the platelet count.



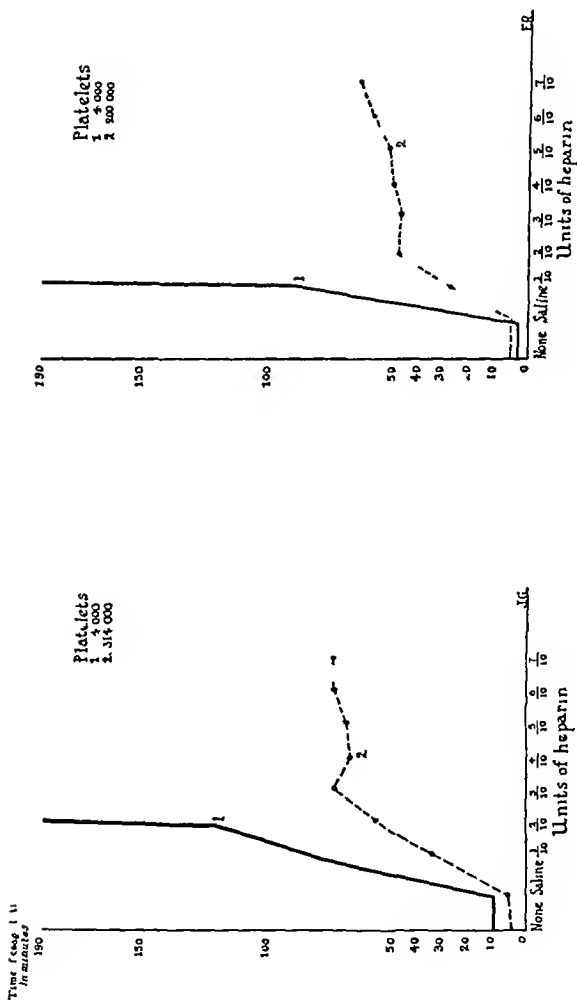


Fig. 4—Showing the Waugh Ruddle curves of four thrombocytopenic patients before and after splenectomy. Note lack of clotting prior to operation.

## SUMMARY

1 The Waugh-Ruddick test has been used to study the coagulation defect in patients with thrombocytopenic purpura

2 The blood from such patients is unusually sensitive to small amounts of heparin *in vitro*

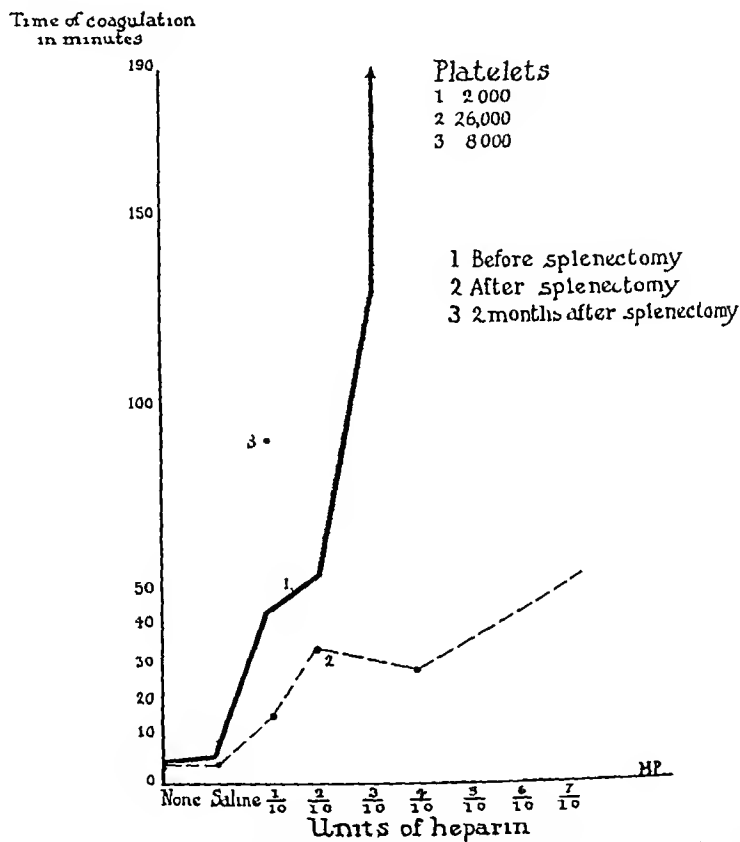


Fig 5—This shows the Waugh-Ruddick curve in a thrombocytopenic patient before splenectomy, one hour postoperatively and two months later at which time bleeding had recurred

3 The increased sensitivity of the blood to heparin parallels closely the clinical manifestations of hemorrhage

4 The coagulation defect does not appear to be due to an excess of heparin in the circulating blood

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II is an exception in that the solution supplied must be diluted before administration. Previous experience in this hospital<sup>9</sup> showed that the solution used was a satisfactory one if administered at a slow rate. All preparations did not have an equal nitrogen content. A number of different lots of each preparation were used to obtain the values listed in Table I. Two lots of Preparation V are listed, the first (Va) consisted of a batch which possessed the highest glutamic acid content of any of this type of mixture. In the second (Vb) a lower glutamic acid content was found and represents an amount closer to the average obtained for this preparation.

TABLE I GLUTAMIC ACID CONTENT OF AMINO ACID PREPARATIONS

PREPARATION	AMINO ACID CONTENT (GM PER CENT)	CONCENTRATION OF AMINO ACID SOLUTION INFUSED (GM PER CENT)	VOLUME INFUSED (ML)	GLUTAMIC ACID CONTENT OF INFUSED SOLUTION (GM)
Preparation II	15	7.5	400	5.05
Preparation I	10	10	500	4.09
Preparation VI	5	5	1,000	2.65
Preparation V				
a	10	10	500	1.16
b	10	10	500	0.25
Preparation III	8	8	500	0.0

A summary of the information obtained by infusing Preparation II is listed in Table II. This preparation contained the highest glutamic acid content (5.05 Gm per 400 cc) of those studied. It can be observed that high serum free glutamic acid levels were reached. Nausea and vomiting was the usual reaction if the glutamic acid content of the blood exceeded 15.9 mg per 100 milliliters. The fact that marked variations in individual tolerance occurs is evident in that one subject did not become nauseated even though he had a serum free glutamic acid level of 42.6 mg per 100 milliliters.

TABLE II THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING  
(PREPARATION II, 5.05 GM GLUTAMIC ACID PER 400 CC OF 7.5% SOLUTION)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (CC/MIN)	REACTION
	BEFORE INFUSION (MG PER CENT)	AFTER INFUSION (MG PER CENT)		
Preparation II				
	4.1	10.6	3.1	0
	5.0	14.1	2.9	0
	3.9	15.9	4.5	0
	3.6	33.0	6.2	Nausea
	3.5	39.0	9.1	Nausea
	3.5	39.0	14.0	Vomited
	4.9	42.6	4.5	0
	4.0	43.0	10.2	Nausea
	2.8	43.2	5.7	Vomited
	--	85.0	3.7	Vomited

In Table III are summarized the results obtained by infusing Preparation I (4.09 Gm glutamic acid per 500 cc). All subjects who had blood free glutamic acid levels above 16 mg per cent became ill. One subject vomited at a lower level (10.9 mg per 100 ml). In general, serum free glutamic acid values were not as high after the administration of this preparation as with the previous one.

TABLE III THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING

(PREPARATION I 4.09 GM GLUTAMIC ACID PER 500 CC)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (CC/MIN)	REACTION
	BEFORE INFUSION (MG PER CENT)	AFTER INFUSION (MG PER CENT)		
Preparation I	3.7	4.8	7.1	0
	4.0	7.3	6.1	0
	7.4	8.2	5.3	0
	4.2	8.8	6.2	0
	0.2	10.7	8.1	0
	4.1	10.8	10.2	0
	4.8	10.9	10.7	Vomited
	--	13.8	7.7	0
	6.6	16.2	8.6	Vomited
	3.9	22.5	5.5	Vomited
	8.3	23.2	0.5	Vomited
	4.4	23.9	9.4	Vomited
	8.0	28.7	5.5	Vomited

Preparation VI (Table IV), an enzymatic digest of bovine blood proteins had a lower free glutamic acid content (2.65 Gm per 1,000 cc) than the previous two preparations and this preparation could be administered at more rapid rates without increasing the toxic symptoms. One subject became nauseated at the rather low serum free glutamic acid level of 9.7 mg per 100 ml, and one subject did not become ill in spite of the high blood level of 22.6 mg per 100 milliliters.

TABLE IV THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING

(PREPARATION VI 2.65 GM GLUTAMIC ACID PER 1000 CC)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (CC/MIN)	REACTION
	BEFORE INFUSION (MG PER CENT)	AFTER INFUSION (MG PER CENT)		
Preparation VI	2.1	4.5	14.7	0
	2.0	6.7	20.2	0
	2.8	6.7	12.5	0
	1.7	7.8	20.0	0
	2.3	9.7	31.2	Nausea
	4.5	9.8	13.3	0
	4.4	10.3	14.3	0
	6.2	13.9	18.2	Vomited
	4.9	10.9	33.3	Vomited
	3.6	22.6	25.0	0

Table V summarizes the data obtained using the preparations containing none or small amounts (1.10 to 0.25 Gm) of glutamic acid. The serum free glutamic acid values obtained following the infusion of 500 ml of an amino acid mixture containing no glutamic acid is of particular interest. In spite of the exceedingly rapid rate of administration (50 and 62 ml per minute), the two subjects showed no toxic symptoms and no appreciable increase in the serum free glutamic acid content. With Preparations Va and Vb attempts were made to produce elevated blood levels by increasing the rate of infusion. It was possible to increase the rate of injection of Preparation Va to such an extent (62.5

ml per minute) that nausea occurred. At the same time the blood free glutamic acid level rose to 12 to 15 mg per 100 milliliters. With Preparation Vb, in only one case of the twelve studied was toxicity noted, but there were no rates of administration which were comparable with the faster infusions given with Preparation Va.

TABLE V THE RELATIONSHIP BETWEEN BLOOD GLUTAMIC ACID LEVEL AND THE PRODUCTION OF NAUSEA AND VOMITING

(PREPARATION VA, 1.10 GM GLUTAMIC ACID, PREPARATION VB, 0.25 GM, AND PREPARATION III, 0.0 GM)

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (CC/MIN)	REACTION
	BEFORE INFUSION (MG PER CENT)	AFTER INFUSION (MG PER CENT)		
Preparation Va	2.2	4.9	12.5	0
	2.7	7.0	14.3	0
	4.7	7.2	20.0	0
	6.2	7.5	13.5	0
	5.0	8.3	15.6	0
	4.1	9.1	16.4	0
	4.0	9.3	35.6	0
	5.5	12.3	62.5	Slight nausea
	--	14.7	62.5	Slight nausea
	4.9	15.7	71.4	Nausea
	4.0	3.0	8.3	0
	3.1	3.7	9.1	0
Preparation Vb	3.8	3.9	16.6	0
	--	4.8	12.5	0
	3.5	4.9	6.3	0
	5.2	5.8	9.6	0
	4.9	6.3	8.8	0
	1.9	6.3	7.1	0
	4.8	6.6	7.1	0
	3.9	7.4	21.7	Vomited
	3.9	7.9	23.8	0
	5.4	9.1	6.0	0
	5.0	5.9	50.0	0
	5.4	6.3	62.5	0
Preparation III				

The data presented in Tables I to V are summarized in Fig. 1. There is good correlation between the serum free glutamic levels and the per cent of subjects who either became nauseated or vomited. Without regard to the preparation used, when the blood free glutamic acid concentration reached 12 to 15 mg per 100 ml, about one-half of the subjects became nauseated or vomited. Above these levels the incidence of ill effects was correspondingly greater.

The subjects of Group C received infusions consisting of Preparation Vb plus 5.05 Gm of glutamic acid per 400 ml of solution (Table VI). The glutamic acid content of the fortified preparation was thus made equal to an acid digest of casein (Preparation II). In this series there was a decidedly greater frequency of toxic symptoms as compared with this same mixture before glutamic acid was added (Table V). With Preparation Vb alone, the incidence of toxic symptoms was 8.3 per cent, with the addition of glutamic acid the incidence rose to 55 per cent. All of the subjects who experienced ill effects had serum free



glutamic acid levels equal to or above the 12 to 15 mg per 100 ml range. It is noteworthy that three of the subjects who had high serum glutamic acid values did not exhibit any gastrointestinal symptoms (Table VI).

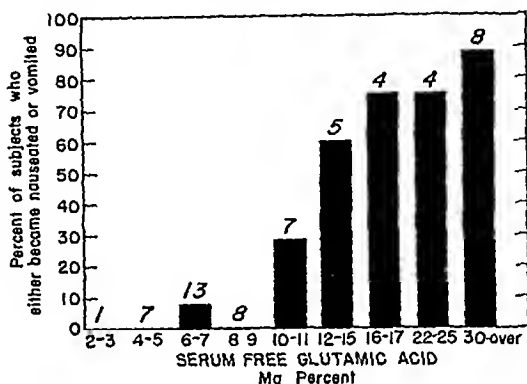


Fig 1

TABLE VI THE EFFECT OF ADDING GLUTAMIC ACID TO PREPARATION Vb ON THE OCCURRENCE OF NAUSEA AND VOMITING

(5.05 GM OF GLUTAMIC ACID ADDED TO 400 CC OF PREPARATION Vb AND STERILIZED BY AUTOCLAVING)

SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (CC/MIN)	REACTION
BEFORE INFUSION (MG PER CENT)	AFTER INFUSION (MG PER CENT)		
2.0	9.0	5.8	0
4.5	9.4	9.3	0
--	11.8	8.0	Nausea
3.4	11.9	8.0	Nausea
3.0	18.0	12.0	Nausea
4.3	19.8	9.3	0
5.9	22.0	10.6	0
5.8	27.6	10.6	0
5.2	29.0	10.6	Nausea
4.8	30.0	9.3	Nausea
7.5	35.0	8.0	Nausea

*Studies Involving Only Glutamic Acid Solutions*—In the next series of studies an attempt was made to determine whether there was a free glutamic acid level of the serum at which the occurrence of nausea and vomiting could be expected (Table VII upper half). The first eight subjects received infusions of solutions containing 5.05 Gm of partially neutralized glutamic acid per 400 ml of solution; the next nine subjects received infusions of a solution containing 10.1 Gm of glutamic acid per 400 milliliters. The level of serum glutamic acid reached was dependent on the concentration of the solution infused and the rate of injection. With the lower concentration none of the eight subjects who received the amino acid exhibited ill effects. Only two of these subjects had

serum free glutamic acid levels exceeding 12 mg per 100 milliliters. Nine subjects received the stronger solution, five of whom became nauseated or vomited. It should be noted that with the stronger solution higher serum free glutamic acid levels were quickly obtained. Forty-five per cent of the subjects who had levels of glutamic acid above 12 mg per 100 ml became nauseated or vomited.

TABLE VII THE RELATIONSHIP BETWEEN SERUM FREE GLUTAMIC ACID LEVELS AND THE OCCURRENCE OF NAUSEA AND VOMITING AFTER GIVING GLUTAMIC SOLUTION INTRAVENOUSLY

AMINO ACID PREPARATION	SERUM FREE GLUTAMIC ACID		RATE OF INFUSION (CC/MIN)	REACTION
	BEFORE	AFTER		
	INFUSION (MG PER CENT)	INFUSION (MG PER CENT)		
<i>Sterilized by Autoclaving</i>				
Glutamic acid 5.05 Gm/400 cc	6.8	5.5	5.3	0
	3.3	6.4	11.0	0
	5.7	7.1	4.0	0
	5.3	8.0	4.6	0
	8.4	8.8	5.3	0
	5.4	11.5	10.0	0
	7.5	16.6	8.0	0
	3.5	35.0	40.0	0
Glutamic acid 10.1 Gm/400 cc	4.3	20.0	9.3	Vomited
	3.1	22.7	8.0	Vomited
	5.7	25.5	9.3	Vomited
	2.7	29.3	10.0	0
	4.0	31.0	12.0	0
	2.0	37.2	10.0	Vomited
	2.1	39.9	15.0	0
	5.9	41.8	10.0	0
	4.1	53.0	9.3	Vomited
<i>Sterilized by Filtering</i>				
Glutamic acid 5.05 Gm/400 cc	3.9	12.0	8.0	0
	3.6	15.1	10.0	0
	3.5	18.0	8.0	0
	3.3	22.6	8.0	Nausea and vomiting
	5.0	26.4	10.6	0
	4.1	27.3	8.0	Nausea
Glutamic acid 10.1 Gm/400 cc	4.4	8.5	6.6	Vomited
	3.3	14.9	8.0	Nausea and vomiting
	2.7	19.3	6.6	Vomited
	2.6	22.4	6.6	Vomited
	4.8	24.0	8.0	Vomited
	6.7	29.7	6.6	Vomited
	2.7	30.9	10.0	Nausea
	2.9	60.0	10.0	Vomited

Since many of the commercial protein digests are sterilized by filtration rather than autoclaving and since heat promotes the conversion of glutamic acid to pyrrolidone carboxylic acid, it was decided to repeat these studies using only glutamic acid solutions which had been sterilized by filtration, employing both the lower and higher doses (Table VII, lower half). When the lower dose was administered, two of the six subjects tested became nauseated or vomited, with the higher doses all of the eight subjects tested became ill. One subject vomited but had a blood glutamic acid level of only 7.5 mg per 100 milliliters.

## DISCUSSION

Until the present investigation the quantitative relationship of glutamic acid to the production of anorexia, nausea, and vomiting following the intravenous administration of casein hydrolysates was based on indirect evidence. Previous studies in this hospital indicated that no significant ill effects attended the giving of amino acid preparations orally, however, a depression of appetite, nausea, or vomiting frequently occurred with or immediately after infusions of casein digests<sup>10</sup>. These ill effects could be reduced to a large extent by reducing the rate of infusion. It was next observed that mixtures of the ten essential amino acids could be given intravenously at very rapid rates without producing ill effects<sup>6, 9, 11</sup>. Both Lekhardt and Davidson<sup>12</sup> and Weinstein<sup>13</sup> have administered intravenously to patients amino acid preparations containing reduced amounts of glutamic and aspartic acids and they have reported few toxic symptoms even with rapid rates of infusion. In the case of the dog<sup>1, 3, 4</sup> (and later confirmed in man), it was noted that the addition of either glutamic or aspartic acid to parenterally administered amino acid mixtures reduced the tolerance of these mixtures. Additional evidence indicated that there was a marked correlation between the content of these two dicarboxylic amino acids in amino acid preparations and the tendency of the solutions to provoke nausea and emesis in man. On the other hand Elman<sup>14</sup> has reported the intravenous injections of an unneutralized glutamic acid solution (1 000 ml of solution contained 30 Gm) into three patients without the occurrence of any ill effects. No reference was made by this investigator to the rate of infusion, or to the glutamic acid level which resulted. His interpretation, apparently based upon that study, that intravenously injected glutamic acid is not related to the production of nausea and vomiting is at variance with our experience.

Hoffman and co-workers<sup>15</sup> reported that if the blood amino acid nitrogen following the administration of an acid hydrolysate of casein exceeds 10 mg per cent, nausea and vomiting may occur. This finding has been confirmed previously in this laboratory<sup>6</sup> for the same preparation; however, using preparations which were either free of or very low in glutamic acid it was possible to obtain markedly elevated blood amino acid nitrogen values without ill effects. At that time it was suggested that the blood glutamic acid probably was more closely related to the nausea and vomiting than the amino acid nitrogen content. Kade<sup>16</sup> has suggested that following the intravenous administration of preparations rich in glutamic acid the blood level of glutamic acid rises faster than the amino acid nitrogen content of the same blood. Friedberg and Greenberg<sup>1</sup> have reported that glutamic acid is slowly cleared from the blood of rats after intravenous administration.

In this study the determination of the free glutamic acid content of serum was accomplished by using a microbiologic procedure employing *Lactobacillus arabinosus* as the test organism. This method has been shown to give reliable values when protein hydrolysates were investigated<sup>7</sup>. Recently it was stated by Prescott and Waelsch<sup>18</sup> that the microbiologic method for glutamic acid is not applicable in the presence of glutamine. By their chemical method it was found

that free glutamic acid of serum or plasma varied from 0.6 to 1.7 mg per 100 ml, and the "apparent glutamine" content ranged from 8 to 12 mg per 100 milliliters. Haipei,<sup>19</sup> using a modified type of microbiologic method, more recently found glutamic acid and glutamine values in plasma similar to those given by the chemical method. The major portion of the preinfusion values for serum free glutamic acid reported in the present study would tend to show that a portion of the serum glutamine is being included in the value, because they are above the 0.6 to 1.7 mg range. Since they are almost all below the 8 to 12 mg range, not all the glutamine is being determined as free glutamic acid.

There appears to be some quantitative difference in the tolerance of glutamic acid when administered alone and when mixed with other amino acids. When 5.05 Gm of glutamic acid were given intravenously as a solution containing only this amino acid, 14 per cent\* (two of the fourteen studied) of the subjects either became nauseated or vomited. When a similar amount of glutamic acid was given intravenously in a mixture of amino acids (Preparation II), 60 per cent\* (six of the ten tested) of the subjects showed ill effects. On the other hand, when the concentration of glutamic acid was increased to 10.1 Gm and only this partially neutralized amino acid was given intravenously, 76 per cent (thirteen of the seventeen patients) of the subjects became nauseated or vomited. Thus, the incidence of nausea and vomiting following the intravenous administration of glutamic acid incorporated in an amino acid mixture is greater than that when an equivalent amount of partially neutralized glutamic acid is given alone. Both aspartic acid<sup>2</sup> and phenylalanine<sup>20</sup> have been reported to increase the toxicity of intravenously administered amino acid. dl Methionine also has been listed as an amino acid which is poorly tolerated when given intravenously to the dog.<sup>21</sup> The amino acid preparations which are low in glutamic acid are also generally low in aspartic acid. Phenylalanine and methionine are probably not offenders in this manner, at least not without the dicarboxylic amino acids being present. Preparations III and V contained these acids and were well tolerated even at rapid rates. The possibility of other amino acids increasing the effect of glutamic acid in the production of nausea and vomiting following its intravenous administration should be further investigated.

In attempting to evaluate the data concerning the relative toxicity of the autoclaved as compared with the filtered solutions of glutamic acid, it was noted that 71 per cent\* (ten of the fourteen cases) of the subjects that received the latter preparation became nauseated or vomited, while only 30 per cent\* (five of the seventeen subjects) became ill after receiving the autoclaved material. Thus, the filtered material would appear to be slightly more toxic than the autoclaved. Olcott<sup>22</sup> reported that if glutamic acid were autoclaved at a temperature of 125° C and in an acid media, pH 3.3 for four hours, 92 per cent of the amino acid would be converted to pyrrolidone carboxylic acid. The possibility of this occurring during the relatively short period of autoclaving used in the present

\*Since the difference is greater than two times the standard error of the mean the differences are considered significant.

study might be an explanation for the apparent difference in emetic effect. The likelihood of the pyrrolidone carboxylic acid assuming like glutamic acid (e.g., supporting the growth of the *Lactobacillus arabinosus*) while having an emetic action different from glutamic acid was investigated, in this case a high serum glutamic acid level might be attained without nausea and vomiting. Lyman found that this microorganism cannot utilize pyrrolidone carboxylic acid.<sup>23</sup> In the present study\* it was found that pyrrolidone carboxylic acid neither stimulated nor inhibited the bacterial growth in that assay of glutamic acid when present in one third to four times the concentration of glutamic acid. If much glutamic acid were converted to pyrrolidone carboxylic acid during the short period of autoclaving then the concentration of free glutamic acid in the autoclaved material should decrease, since the pyrrolidone carboxylic acid does not support growth of the microorganism. This did not occur. Hence, it seems unlikely that pyrrolidone carboxylic acid could account for the apparent difference in toxicity of the filtered and autoclaved glutamic acid solutions.

#### SUMMARY AND CONCLUSIONS

The free glutamic acid content of five different types of amino acid preparations was estimated using microbiologic methods. They are, in decreasing order of glutamic acid content: an acid hydrolysate of casein fortified with tryptophane, an enzymatic hydrolysate of casein, an enzymatic digest of beef blood proteins, a mixture of amino acids produced by the recombination and fortification of fractions of a casein digest, and a mixture of the ten "essential" amino acids plus glycine. The occurrence of nausea and vomiting following the administration of the various preparations in a series of fifty-seven human subjects was found to parallel the free glutamic acid content of the mixture given.

Until the present investigation only indirect evidence was obtained concerning the quantitative relationship of glutamic acid and the production of nausea and vomiting following its intravenous administration. A direct relationship between the free glutamic acid of the serum and the occurrence of toxic effects following the administration of amino acid preparations was found in this investigation. When the serum free glutamic acid level reached 12 to 15 mg per 100 ml, nausea and vomiting occurred in more than half of the subjects.

Solutions containing only partially neutralized glutamic acid were administered intravenously to thirty-one individuals and estimates of the free glutamic acid content of the serum were made. A lower percentage of these patients became ill at elevated glutamic acid levels than in the group that received glutamic acid containing amino acid mixtures. This is interpreted as showing that the toxicity of glutamic acid may be potentiated by other amino acids.

A study of the effect of autoclaving and of sterilization by filtration upon the toxicity of glutamic acid solutions indicated that the filtered material was slightly more toxic. This difference could not be attributed to the presence of pyrrolidone carboxylic acid.

\*These data are not included in the present report.

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# CARBONIC ANHYDRASE ACTIVITY IN SICKLE CELL ANEMIA, SICKLE CELL TRAIT, AND PLEUROCIOUS ANEMIA

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CARBONIC anhydrase an enzyme first described by Meldrum and Roughton<sup>1</sup> and Stille and O'Brien<sup>2</sup> is present in the erythrocytes of homeothermic animals and in the gills of some poikilothermic ones.<sup>3</sup> It plays an important role in the transport and exchange of CO<sub>2</sub>. No studies have been reported previously on the presence of carbonic anhydrase in sickle cell anemia, although this enzyme has been implicated in the sickling phenomenon by Tomlinson and Jacob.<sup>4</sup> These authors found that washing erythrocytes from patients with sickle cell anemia or sickle hemia twenty times in normal saline almost always removes their ability to assume sickled forms in normal saline. They suggested that the multiple washings remove some substance, possibly carbonic anhydrase the presence of which is necessary for the phenomenon of sickling. Additional evidence for this hypothesis was presented by the demonstration that certain carbonic anhydrase inhibitors such as zinc acetate and sodium cyanide in concentrations of 1/1000 when added to oxygenated blood prohibit sickle cell formation; however, other carbonic anhydrase inhibitors namely sulfanilamide and immanium thiocyanate give inconsistent results.

In the present studies the relationship between carbonic anhydrase and the sickling phenomenon was investigated by the following means: (1) The carbonic anhydrase content of the blood of patients with sickle cell anemia and with sickle cell trait was compared with that of normal individuals. Three cases of pleurocious anemia were included for comparison. (2) Erythrocytes from patients with the sickling tendency were washed repeatedly in normal saline, until sickling no longer occurred; the red blood cell suspension was then made up to its original volume with water and again tested for the presence of carbonic anhydrase. The saline washings also were tested for the presence of carbonic anhydrase. (3) The susceptible erythrocytes were washed repeatedly until sickling no longer occurred and a concentrated solution of human carbonic anhydrase was added to determine whether such addition restored the ability of the cells to sickle.

## METHODS

Carbonic anhydrase determinations were made by the colorimetric method of Philpot and Philpot<sup>5</sup> and enzyme units were estimated according to this method. Each determination was made in triplicate. Oxalated blood was used throughout. Appropriate dilutions

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were made of whole blood, except in those experiments in which washed cells were used. Concentrated carbonic anhydrase preparations were made by treating the washed and hemolyzed red blood cells with a mixture of alcohol and chloroform and centrifuging off the precipitate. The carbonic anhydrase activity of the supernatant fluid was about fifty times that of the whole blood.

TABLE I CARBONIC ANHYDRASE CONTENT OF BLOOD OF NORMAL INDIVIDUALS AND THOSE WITH SICKLE CELL ANEMIA, SICKLE CELL TRAIT, AND PERNICIOUS ANEMIA

1 CARBONIC ANHYDRASE CONTENT OF BLOOD OF NORMAL INDIVIDUALS				
DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C MM)	HEMOGLOBIN (GM/100 CC)	CARBONIC ANHYDRASE (ENZYME UNITS/C MM)
1/7	V J P	4.57	14.4	1,220
1/7	G M	5.09	16.7	1,240
1/7	F S	4.42	12.7	960
3/4	B L	4.92	17.0	846
3/16	I B	5.82	16.5	814
3/16	P T	5.7	16.5	1,380
4/14	L J	4.2	12.7	1,280
4/18	M S	4.5	13.0	1,026
4/27	S	4.14	12.7	1,140
Average		4.81	14.68	1,100
2 CARBONIC ANHYDRASE CONTENT OF BLOOD OF PATIENTS WITH SICKLE CELL ANEMIA				
DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C MM)	HEMOGLOBIN (GM/100 CC)	CARBONIC ANHYDRASE (ENZYME UNITS/C MM)
3/23	A R	2.41	6.7	670
3/31	W S	4.17	9.4	692
3/31	M H F	3.48	9.4	780
4/4	P P	2.87	10.5	732
4/7	W B	3.0	10.4	814
4/27	O W	2.27	7.0	940
Average		3.03	8.9	771
Per cent of normal		62	60	71
3 CARBONIC ANHYDRASE CONTENT OF BLOOD OF INDIVIDUALS WITH SICKLE CELL TRAIT				
DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C MM)	HEMOGLOBIN (GM/100 CC)	CARBONIC ANHYDRASE (ENZYME UNITS/C MM)
1/7	L P	3.58	11.3	1,120
1/7	C J	4.51	12.1	1,020
1/7	I C	4.08	12.7	1,020
1/7	N B	4.55	14.6	980
4/4	G M	4.1	13.2	1,060
4/14	L G	3.66	9.4	1,260
4/27	E C	4.68	14.0	960
Average		4.76	12.4	1,060
Per cent of normal		86	84	96
4 CARBONIC ANHYDRASE CONTENT OF BLOOD OF INDIVIDUALS WITH PERNICIOUS ANEMIA				
DATE	SUBJECT	RED BLOOD CELLS (MILLIONS/C MM)	HEMOGLOBIN (GM/100 CC)	CARBONIC ANHYDRASE (ENZYME UNITS/C MM)
1/4	T B L	2.21	9.1	680
1/4	S	2.03	9.3	1,020
3/16	T	1.23	3.3	680
Average		1.92	7.23	1,000
Per cent of normal		38	49	91



Sickling of cells was estimated by examining sealed wet smears of blood after incubation at room temperature for twenty four hours. The erythrocytes were washed twenty times with normal saline according to the technique described by Tomlinson and Jacob.<sup>4</sup> In certain instances carbonic anhydrase was added to whole blood and to cells washed five, ten, fifteen and twenty times. The amount of sickling after such treatment was compared with that of untreated cells.

## RESULTS

In Table I is given the carbonic anhydrase content of the blood of individuals with sickle cell anemia and sickle cell trait as compared with normal individuals and three persons with pernicious anemia. In Table II these data are summarized in the form of ratios of carbonic anhydrase to red blood cell count and hemoglobin content. These ratios indicate that the carbonic anhydrase content of red blood cells with sickling capacity is as high as or

TABLE II RATIO OF BLOOD CARBONIC ANHYDRASE TO RED BLOOD CELLS AND HEMOGLOBIN OF NORMAL INDIVIDUALS AND THOSE WITH SICKLE CELL ANEMIA, SICKLE CELL TRAIT, AND PERNICIOUS ANEMIA

NUMBER OF PATIENTS	DIAGNOSIS	RATIO OF AVERAGE CARBONIC ANHYDRASE TO AVERAGE RBC COUNT	RATIO OF AVERAGE CARBONIC ANHYDRASE TO AVERAGE HEMOGLOBIN CONCENTRATION
9	Normal	1.228	1.74
6	Sickle cell anemia	1.254	1.87
7	Sickle cell trait	1.223	1.85
3	Pernicious anemia	1.540	1.138

slightly higher than that of normal red blood cells. In pernicious anemia, red blood cells contain much larger quantities of carbonic anhydrase, although only three cases of pernicious anemia were studied the difference appears striking.

Table III summarizes the data obtained by determining the carbonic anhydrase content of cells with the sickling capacity washed twenty times in isotonic saline until sickling no longer occurred. Ninety per cent of the carbonic anhydrase content of the original whole blood is present in these washed cells. The saline washings contain only a trace of carbonic anhydrase, one determination of the carbonic anhydrase activity of the plasma of these washed cells is noted. Part of the carbonic anhydrase lost by washing is apparently contained in the plasma where it doubtless appears because of hemolysis.

TABLE III CARBONIC ANHYDRASE CONTENT OF RED BLOOD CELLS AFTER WASHING UNTIL SICKLING CAPACITY IS REMOVED

PATIENT	DIAGNOSIS	CARBONIC ANHYDRASE WHOLE BLOOD (ENZYME UNITS/C MM)	CARBONIC ANHYDRASE WASHED CELLS (ENZYME UNITS/C MM)	CARBONIC ANHYDRASE SALINE WASHINGS	CARBONIC ANHYDRASE CONTENT OF PLASMA (ENZYME UNITS/C MM)
I. P.	Sickle cell anemia	732	703	—	—
O. W.	Sickle cell anemia	940	840	Trace	—
F. C.	Sickle cell trait	960	840	Trace	76
Average		847	796		

Table IV illustrates the results of adding concentrated carbonic anhydrase solution to cells from which the sickling capacity has been removed by repeated washing in isotonic saline. Such addition of carbonic anhydrase does not restore the sickling capacity of the cells.

TABLE IV EFFECT OF CARBONIC ANHYDRASE ON THE ABILITY OF SUSCEPTIBLE CELLS TO SICKLE AFTER MULTIPLE WASHINGS WITH ISOTONIC SOLUTION OF SODIUM CHLORIDE

DATE	PATIENT	DIAGNOSIS	NUMBER OF WASHINGS	PER CENT SICKLING OF UNTREATED CELLS	PER CENT SICKLING AFTER CONCENTRATED CARBONIC ANHYDRASE ADDED	PER CENT SICKLING AFTER ADDITION OF CARBONIC ANHYDRASE DILUTED 1:10 WITH 0.85% NaCl
2/11/49	V B	Sickle cell anemia	0	9.6		16
			5	0		0.6
			10	0		6.0
			15	0		0
			20	0		0
2/10/49	M F	Sickle cell anemia	0	82.8		83.2
			5	80		78
			10	60.8		0
			15	25.6		37.4
			20	28.2		17.4
2/10/49	A A	Sickle cell anemia	0	75.4		80.4
			5	0		0
			10	0		0
			15	0		0
			20	0		0
2/18/49	R A	Sickle cell anemia	0	92.2		91
			5	62		77.3
			10	12.2		6.4
			15	13.6		Hemolyzed
			20	13.0		Hemolyzed
3/7/49	A R	Sickle cell anemia	0	Hemolyzed	15.6	8.4
			5	10	Hemolyzed	15.8
			10	7.2	3.9	5.4
			15	13.6	10	10.9
			20	12	30.2	9.8

#### DISCUSSION

Our results indicate that the loss of sickling capacity of red blood cells from patients with sickle cell anemia or sickle cell trait is not associated with a significant loss of carbonic anhydrase. After the completion of these experiments there appeared in *Blood* an editorial by Vallee and Altschule<sup>6</sup> referring to a series of papers in press on carbonic anhydrase in anemia. In this editorial and in an abstract recently published by Vallee<sup>7</sup> a striking difference is drawn between the carbonic anhydrase content of red blood cells from patients with pernicious anemia and those from patients with secondary anemias and from normal individuals. Our findings indicate that blood from sickle cell anemia and sickle cell trait is similar to that of secondary anemia in this respect. In contrast, our three cases of pernicious anemia have twice the ratio of carbonic anhydrase to red blood cells and hemoglobin compared with the normal values.

## SUMMARY

1 The carbonic anhydrase content of red blood cells exhibiting the sickling phenomenon corresponds to that of normal red blood cells

2 Washing red blood cells of individuals with sickle cell anemia or sickle cell trait with isotonic saline until sickling no longer occurs results in no significant loss of carbonic anhydrase activity

3 Addition of carbonic anhydrase to washed cells from which the sickling capacity has been removed does not restore this capacity

4 Carbonic anhydrase, under conditions described, has no effect on the sickling phenomenon

5 Confirmatory evidence is presented that in pernicious anemia unit values of carbonic anhydrase in respect to red blood cell count and hemoglobin are about twice those of normal individuals

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## CLINICAL STUDIES ON THIOMERIN, A NEW MERCURIAL DIURETIC

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SEVERAL effective mercurial diuretics are available and are widely used at the present time. The introduction of a new diuretic can be justified only if it is less toxic or more effective, or if it is more convenient to use. This report concerns itself with the results of a study of a new mercurial diuretic whose toxicity and efficiency have been compared with those of several of the preparations in common usage. The new material is the disodium salt of N( $\gamma$ -carboxymethyl mercaptomercuri  $\beta$ -methoxy) propyl camphoramic acid (Thiomein) \*. It has the same organic-mercurial structure as Meleuzanthum, with the theophylline replaced by sodium mercaptoacetate. Thiomein is a white amorphous powder which is supplied in vials containing 1.4 Gm. of the compound. The introduction of 10 cc. of sterile water into the vial results in a solution containing the equivalent of 0.040 Gm. of mercury per cubic centimeter at a pH of about 7.5.

## METHODS

The method of study was similar to that employed by DeGraff.<sup>1</sup> Patients in congestive heart failure and with considerable edema were digitalized and kept at bed rest on a relatively salt poor diet (approximately 3 grams of sodium chloride daily). There was no limitation of fluid intake. Each patient's weight was recorded daily at the bedside until there was no further weight loss over the course of several days. At this point the diuretic was administered. Patients who were too ill to be weighed or whose weight might have been affected by variation in dietary intake, vomiting, decrease in hyperthyroid state, or by factors other than edema were not included in this study. Each patient's urine was examined for abnormalities before and after the administration of the diuretic. Injections of Thumerin were given subcutaneously, except in two instances when it was administered intravenously. The injections of Mercuzanthin and of Salyrg in Theophylline were given intramuscularly.

## RESULTS

The diuretic effect of Thiomein in seventy trials on thirty-six patients is indicated in Fig 1. There was diuresis resulting in weight losses ranging from 2 to 17½ pounds. This range of weight loss was roughly proportional to the amount of edema present. In all the Thiomein trials (in which weight loss was studied) the average weight loss was 6 pounds and the median weight loss was 5 pounds, while somewhat less than 50 per cent of injections resulted in a weight loss between 3 and 4 pounds. The diuretic effect usually appeared within several hours after the injection and lasted for twenty-four hours or more. The duration of action varied. Usually it was over by the end of twenty-four hours, although in several patients it lasted for three days. Variation in duration of

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\*Thiomerin has the following structural formula

$$\text{CH}_3\text{CH}_2\text{CHCONHCH}_2\text{CH}(\text{OCH}_2\text{CH}_2\text{HgSCH}_2\text{COONa})\text{CH}_3$$

action sometimes occurred in the same patient from one injection to the next. In no instance was there any evidence of any nephrotoxic effect. From these results it can be seen that Thiomerein is an effective diuretic.

The diuretic effect of Thiomerein was next compared with that of two widely used mercurial diuretics, Mercuzanthin and Salyrgan. Theophylline. Approximately the same amount of mercury (0.04 Gm.) is present in 1 c.c. of all these preparations.

#### DIURETIC EFFECT OF THIOMERIN IN 70 TRIALS

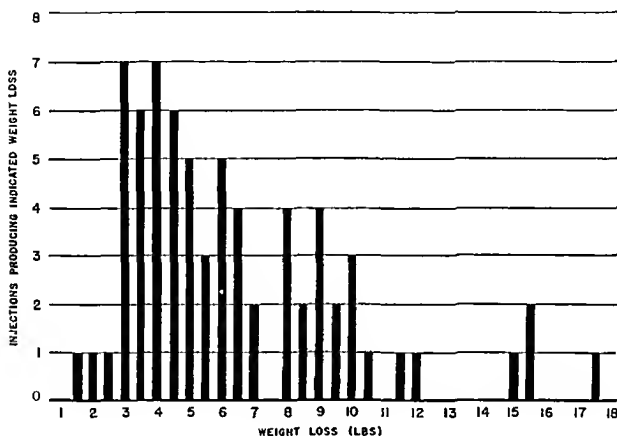


FIG. 1

As shown in Fig. 2, nineteen comparisons of the diuretic activity of Thiomerein and Salyrgan Theophylline were made in thirteen patients. In seven instances (37 per cent) the diuretic effect was the same. In nine instances (47 per cent) Thiomerein produced a greater diuresis and in three instances (16 per cent) Salyrgan Theophylline had a greater effect. The duration of diuretic action was also noted for the two drugs. In six instances the Thiomerein induced diuresis was more prolonged; in two instances a more sustained effect was induced by Salyrgan Theophylline. In Case 44 Thiomerein induced diuresis in two trials was more prolonged and greater than that obtained with Salyrgan Theophylline. This was true despite the fact that the Thiomerein was given intravenously on one occasion and subcutaneously on the next.

The relative diuretic action of Thiomerein and Mercuzanthin was observed in ten comparisons on eight patients. In five of these instances the diuresis obtained was the same; in three it was greater with Thiomerein and in two it was greater with Mercuzanthin. It also was noted that in three instances Thiomerein induced diuresis was slower and more prolonged.

*Toxicity*—Urinalyses before and after the administration of Thiomerin did not reveal evidence of renal toxicity. This was true in a patient with chronic glomerulonephritis as well as in the patients with heart disease of other etiology.

COMPARISONS OF DIURETIC EFFECT OF THIOMERIN,  
MERCUZANTHIN AND SALYRGAN-THEOPHYLLINE  
INDIVIDUAL CASES

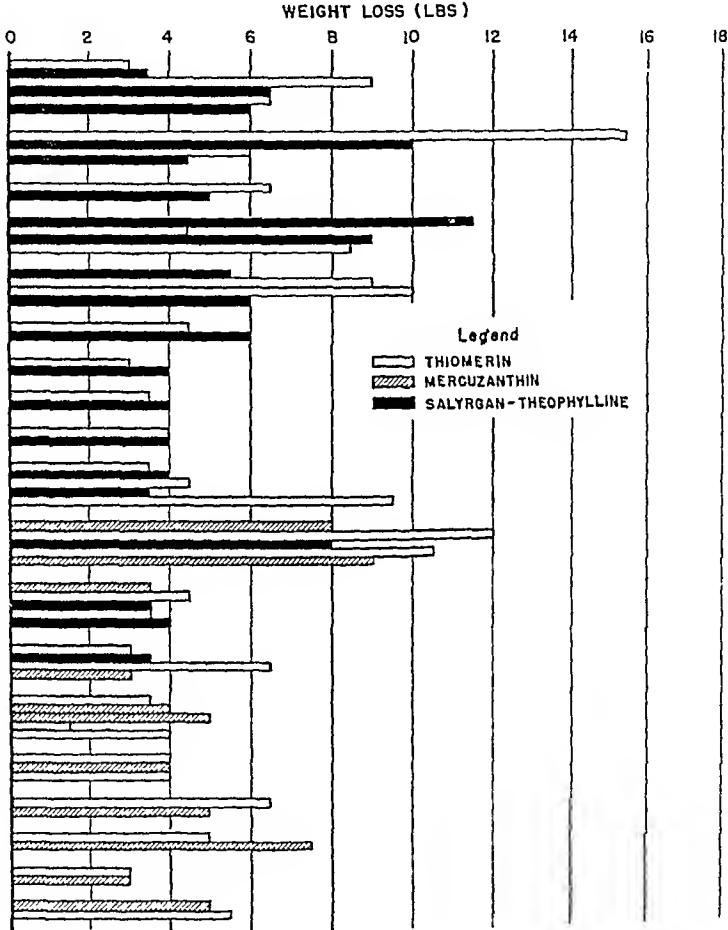


Fig. 2

Since Thiomerin is given subcutaneously, local effects were watched for. Immediate reactions were minimal. They consisted of slight burning or stinging at the site in 41 per cent of the injections. A small number of patients also had some aching or local soreness lasting a few minutes to a few days. Because a certain number of nodules or infiltrations were found to follow use of the drug, it was decided to study this long-term phenomenon more carefully in another group of patients. While the early product, no longer being manufactured, caused appreciable indurations in 12 per cent of injections, the drug as

it is now being prepared gave rise to such infiltrations only four times (3.3 per cent) in a total of 121 injections. These areas, which were similar after both products, were situated subcutaneously and could usually not be outlined clearly since they faded into the surrounding tissue. They were about  $\frac{1}{4}$  in to  $1\frac{1}{2}$  in in diameter, were up to  $\frac{1}{2}$  in in depth, were occasionally attached to the skin, and were only rarely tender. They cleared up gradually in a period of two to five months. Biopsies done on four of these infiltrations showed them to consist of areas of aseptic fat necrosis. In this last series of 121 injections there were also six instances (5 per cent) of local infiltrations so small that they would have been missed had they not been searched for carefully.

#### DISCUSSION

With the extensive use of mercurial diuretics, a number of reports have appeared which emphasize fatal or near fatal reactions from their intravenous use.<sup>3,4</sup> The possibility of such toxic reactions has been present regardless of the mercurial preparation employed. It is due to direct action on the heart with the production of disturbances in conduction and rhythm<sup>5</sup> and in working capacity.<sup>7</sup> A recent study in animals of the comparative toxicity of the commonly used mercurial diuretics and of Thiomerin has shown the latter to be 160 to 200 times less toxic to the heart.<sup>8</sup> No effect was seen by electrocardiogram in anesthetized cats after a dose of 4 cc of Thiomerin per kilogram, as compared with 0.1 cc per kilogram of other mercurial diuretics. If these results may be transposed to man this would compare a dose of 240 cc of Thiomerin to one of 6 cc of Mercuzanthin, Salyrgan, Theophylline, or Mercurhydrin. Thus the danger of sudden cardiac death would seem to be practically eliminated.

Our results confirm the findings of Boig and Craig<sup>9</sup> and Hermann and co-workers<sup>10,11</sup> as to the efficacy of Thiomerin as a diuretic for subcutaneous administration. The diuresis obtained by us following the subcutaneous injection of Thiomerin was comparable with that obtained with equivalent amounts of Mercuzanthin and Salyrgan Theophylline given intramuscularly. In some patients it was a more effective diuretic. The effect was more sustained in a number of instances. This may be related to the site of administration, although in one patient the intravenous injection of Thiomerin produced more sustained diuresis than did an intramuscular injection of Salyrgan Theophylline.

#### SUMMARY

1 The diuretic effectiveness of Thiomerin, a new mercurial compound which is reported to be less toxic to the heart when administered intravenously to cats, has been investigated in man.

2 Thiomerin was found to be an effective diuretic in seventy trials on thirty-six patients.

3 The diuresis obtained after the subcutaneous injection of 2 cc of Thiomerin was comparable with or greater than that noted after the intramuscular use of equivalent amounts of Mercuzanthin or Salyrgan Theophylline in twenty-nine comparative studies on twenty-one patients. In some patients the diuresis with Thiomerin was more sustained.

4 No systemic toxic effects were noted Kidney damage, as judged by urine examinations, did not occur

5 Local reactions following the subcutaneous use of Thiomerin were absent or insignificant 96.7 per cent of the time Three and three tenths per cent of injections were followed by annoying but seemingly harmless subcutaneous infiltrations of long duration

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## LIMITATIONS AND MERITS OF A SINGLE SERUM SAMPLE ANALYSIS IN THE DIFFERENTIAL DIAGNOSIS OF JAUNDICE

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### INTRODUCTION

PREVIOUS experiences in the employment of multiple biochemical procedures to assay liver function and to assist in determining the cause of jaundice have been reported from this clinic<sup>1</sup>. The differential diagnosis of jaundice is a problem that can be solved only by the exercise of sound clinical judgment at the patient's bedside. Laboratory studies are at best only auxiliary aids to this problem. Until we possess specific biologic tests for the recognition of virus hepatitis the detection of this disorder depends chiefly upon inferences drawn from the history of the illness, the physical examination and the laboratory evidence of liver dysfunction. Since we lack a non-operative roentgenologic method of visualizing the extrahepatic biliary tract in the jaundiced patient the diagnosis of obstruction due to calculi, to tumors, or to stricture must similarly be based upon inferences from such data.

In 1945 Schwimmer, Klotz, Dieker and McGavack<sup>2</sup> described their extensive experience with a fasting blood sample in the differential diagnosis of jaundice. They pointed out the many practical advantages of this procedure. The single specimen group of tests employed by these investigators included the icteric index, the van den Bergh reaction, the cephalin cholesterol flocculation test, the serum alkaline phosphatase value, the total and esterified serum cholesterol, and the total serum proteins together with the albumin and globulin fraction. The application of these tests to a wide variety of hepatic disorders by Schwimmer and associates revealed that the composite test yielded results that were as delicate as accurate and as informative as those derived from the more elaborate tests of liver function.

Shay and Siplet<sup>4</sup> stated that a 'minimum yet adequate liver function study' may be made in jaundice with the following tests: (1) quantitative van den Bergh, (2) serum cholesterol partition, (3) serum alkaline phosphatase, (4) thymol turbidity and (5) cephalin cholesterol flocculation. They illustrated the merits of these tests when applied serially to individual cases. More recently Popper and Steigmann<sup>5</sup> have evaluated the merits of these tests as well as others in current usage in 285 patients. The initial series of liver function tests was taken into consideration in that study.

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The present study was made to test the accuracy with which a group of tests performed on a *single* fasting serum sample can assist in the differential diagnosis in a jaundiced patient. It is not our contention that the analysis of a single specimen of serum from a jaundiced patient should be entirely adequate. Serial observations are certainly indicated and were, in fact, carried out in the majority of our patients. These often included tests other than those to be discussed in this paper.

#### MATERIALS AND METHODS

In the selection of cases for the present study only instances of regurgitation jaundice have been chosen for analysis. Instances of retention jaundice usually have other distinguishing features and seldom present the diagnostic problems that regurgitation jaundice does. One hundred forty seven patients observed at the University of Minnesota Hospital were included. The following criteria were employed in the selection.

1 *Presence of jaundice* The study has been limited to instances in which the prompt direct reacting component (1 min. value) of the serum bilirubin was greater than 0.4 mg per 100 cc and the total bilirubin greater than 2.0 mg per 100 cubic centimeters.

2 *Available data* Evaluation has been limited to instances in which the following determinations were made on a *single* sample of serum secured in the fasting state: bilirubin, total and esterified cholesterol, alkaline phosphatase, thymol turbidity, and cephalin cholesterol flocculation.

3 *Proof of diagnosis* Evaluation has been limited to patients in whom the diagnosis was established by operation, necropsy, or biopsy except in the cases of virus hepatitis where a clinical diagnosis was accepted despite lack of biopsy data. A critical appraisal of the available clinical material was made and the selection was restricted to instances in which the course of the illness was believed to establish the diagnosis beyond reasonable doubt.

Of the 147 patients, seventy seven had extrahepatic biliary obstruction and seventy suffered from parenchymal liver disease. The former group included:

- 1 Twenty eight cases of carcinoma obstructing the bile ducts
- 2 Thirty nine cases of common duct calculus
- 3 Ten cases of stricture of the bile ducts

In each instance the diagnosis was verified by operation or by necropsy. The group of cases of parenchymal jaundice included:

- 1 Twenty six cases of cirrhosis
- 2 Thirty one cases of virus hepatitis
- 3 Thirteen cases of liver disease due to miscellaneous causes

The methods employed are listed in Table I. In three cases (see Table VI) and in one case (Table VII) the thymol turbidity was performed according to the colorimetric adaptation of Ducci.<sup>12</sup> The upper limit however is the same as by the MacLagan method.<sup>13</sup>

TABLE I BIOCHEMICAL PROCEDURES EMPLOYED

PROCEDURE	METHOD	NORMAL VALUE
1 Serum bilirubin	Malloy and Evelyn <sup>11</sup> as modified by Ducci and Watson <sup>12</sup>	1* (prompt direct) 0.2 mg / 100 cc (total, direct plus indirect) 1.0 mg / 100 cc
2 Cephalin cholesterol flocculation	Hanger <sup>8</sup>	1 reading greater than 1+ at 24 hr may be considered abnormal
3 Thymol turbidity	MacLagan <sup>9</sup>	0 - 4 units
4 Serum cholesterol (total)	Schoenheimer and Sperry <sup>10</sup>	180 - 220 mg per 100 cc
5 Serum cholesterol (esterified fraction)	Schoenheimer and Sperry <sup>10</sup>	50 - 65% of total
6 Alkaline phosphatase	Bodansky <sup>11</sup>	1 - 4 units per 100 cc

## RESULTS

The values presented in Table II indicate the anticipated results if one were to assume that these procedures would always yield results of differential diagnostic value. The values given are admittedly arbitrary. It is well known that certain cases of diffuse liver disease exhibit functional changes that are very similar to those produced by mechanical obstruction of the extrahepatic bile ducts. Such instances may be considered examples of cholangiolar dysfunction. However, the majority of cases of hepatitis and cirrhosis might be expected to exhibit hepatocellular dysfunction and to conform therefore to anticipated results insofar as these tests are concerned.

The actual values obtained in the different types of jaundice are given in Tables III to VIII. The cases have been arranged in order of the increasing duration of jaundice insofar as the duration could be ascertained from the history. The numbers in italics in these tables indicate instances in which the actual results fail to conform with those listed in Table II as anticipated results. These data have been tabulated in Table IX.

TABLE II ANTICIPATED RESULTS IN BIOCHEMICAL TESTS EMPLOYED IN PRESENT STUDY

TYPE OF LIVER DISEASE	CEPHALIN CHOLESTEROL (24 HR READING)	THYMOL TURBIDITY (MACLEOD'S)	TOTAL SERUM CHOLESTEROL	CHOLESTEROL ESTER (PERCENTAGE)	ALKALINE PHOSPHATASE (BODANSKY)
Extrahepatic obstruction	1+ or below	0 - 4 units	Above 225 mg per 100 cc	Above 50%	Above 10 units
Parenchymal liver disease	Above 1+	Above 4 units	Below 225 mg per 100 cc	Below 50%	Below 10 units

*Bilirubin*—In Tables III through VIII, the relationship of the one minute serum bilirubin to the total has been computed as percentage. The data for the serum bilirubin are presented in graphic form in Fig 1 where the cases are arranged in order of increasing duration of jaundice. No consistent pattern of behavior is discernable.

*Serum Cholesterol*—An analysis of Table IX reveals that the total serum cholesterol was below 225 mg per 100 cc in twenty instances in the seventy seven cases of extrahepatic biliary obstruction. Furthermore, the cholesterol esters constituted less than 50 per cent of the total in thirty four cases in this group. The cholesterol ester fraction was below 50 per cent in twenty one of the twenty eight cases in the carcinoma group.

The cholesterol levels failed to correspond with the anticipated results in twenty two of the seventy cases of parenchymal liver disease. The thirty one cases of hepatitis included in these seventy cases represent a more homogeneous group. Even so eleven of these thirty one patients had cholesterol levels greater than 225 mg per 100 cc at the time the multiple tests were performed. Nine of these eleven patients had ester fractions which comprised more than 50 per cent of the total cholesterol.

TABLE III RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO CALCULI

CASE	SEX	AGE (yr.)	DURATION OF ILLNESS	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN CHOLESTEROL		THYMOL TURBIDITY	ALBA LINE PHOS. TAPE	DIAGNOSIS ESTABLISHED BY
				1	TOTAL	1/T %	TOTAL	ESTER	ESTER %	24 HR	48 HR			
1	M	77	2 days	1.0	2.2	45	174	92	53	0	0	2	32	Operation
2	F	83	6 days	4.2	7.4	57	288	46	16	5+	4+	6	25	Necropsy
3	F	81	7 days	17.5	30.7	57	360	50	14	Tr	1+	2	50	Necropsy
4	M	71	10 days	9.9	17.0	58	354	103	29	0	0	2	18	Operation
5	M	56	12 days	9.2	15.2	61	198	115	58	2+	3+	1	31	Necropsy
6	M	60	14 days	3.8	6.2	61	258	218	61	0	1+	3	6	Operation
7	M	40	14 days	9.9	18.1	55	234	133	57	1+	1+	3	6	Operation
8	M	66	3 wk.	13.7	24.0	57	350	126	36	0	0	3	22	Operation
9	M	70	4 wk.	8.0	12.1	66	114	80	26	1+	1+	3	12	Necropsy
10	F	71	4 wk.	15.6	27.9	55	676	277	41	0	0	3	69	Necropsy
11	F	60	4 wk.	14.5	25.4	57	594	184	31	0	Tr	2	24	Operation
12	F	62	4 wk.	8.0	18.2	47	570	274	48	0	0	2	22	Operation
13	F	75	5 wk.	9.0	14.5	62	238	74	31	0	1+	2	13	Necropsy
14	M	63	6 wk.	9.9	17.5	57	276	125	53	0	1+	2	30	Operation
15	M	68	6 wk.	18.7	63.5	29	310	46	15	1+	1+	2	19	Operation
16	M	59	6 wk.	24.0	41.6	58	506	40	8	0	0	1	31	Necropsy
17	M	56	6 wk.	9.4	20.7	45	236	47	20	1+	1+	5	31	Necropsy
18	M	61	6 wk.	3.3	7.1	46	268	185	69	0	Tr	2	12	Operation
19	M	74	7 wk.	17.0	31.7	57	290	107	37	0	0	1	18	Operation
20	M	65	7 wk.	16.8	31.0	54	640	214	33	0	0	2	32	Necropsy
21	F	67	7 wk.	12.8	22.6	57	510	56	11	0	0	1	35	Operation
22	M	72	8 wk.	1.9	3.6	53	208	125	60	1+	1+	2	13	Operation
23	F	62	8 wk.	15.3	26.0	59	360	83	23	1+	1+	3	20	Necropsy
24	F	60	4 mo.	11.8	28.3	52	296	15	5	Tr	1+	2	15	Necropsy
25	M	76	4 mo.	15.8	28.8	55	130	32	25	1+	1+	5	44	Necropsy
26	F	72	6 mo.	17.7	31.8	56	730	346	48	1+	1+	3	27	Operation
27	F	56	11 mo.	8.7	11.5	60	362	98	27	0	0	4	9	Operation
28	M	35	?	10.1	17.1	60	279	100	27	0	0			

TABLE IV RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO COMMON DUCT STONE

CASE	SEX	AGE (Y)	DURATION OF JAUNDICE	SERUM BILIRUBIN		CHOLESTEROL			CERIALIN CHOLESTEROL		TH3 MOL TUB BID ITY	ALAA LINE PHOS PIA TASE	DIAGNOSIS ESTABLISHED BY
				I	TOTAL	1/%	TOTAL	ESTER	ESTER-%	24 HR	48 HR		
29	F	77	2 days	10	24	42	240	154	64	0	0	9	Operation
30	F	68	2 days	47	82	57	100	29	29	1+	1+	6	Necropsy
31	F	73	2 days	10	21	48	328	216	66	1+	1+	25	Operation
32	F	73	3 days	25	49	51	164	82	50	0	Tr	5	Operation
33	F	62	3 days	16	32	50	194	116	60	0	1+	24	Operation
34	F	67	4 days	70	114	61	134	41	38	1+	2+	8	Operation
35	M	41	4 days	21	39	54	272	169	62	0	0	35	Operation
36	M	81	5 days	18	32	55	234	140	63	0	0	18	Necropsy
37	F	67	5 days	13	28	46	250	175	70	0	0	26	Operation
38	F	36	7 days	80	141	57	240	84	35	8+	4+	14	Operation
39	M	64	7 days	12	22	54	156	107	71	1+	2+	15	Operation
40	M	73	8 days	20	48	60	126	79	63	0	0	5	Operation
41	M	74	14 days	54	129	65	328	167	51	0	0	3	Operation
42	F	31	14 days	78	118	60	338	98	29	0	0	15	Operation
43	F	37	3 wk	09	26	35	160	107	67	0	0	6	Operation
44	F	54	3 wk	04	97	50	230	62	27	0	Tr	12	Operation
45	M	57	3 wk	60	86	63	192	117	61	1+	1+	34	Operation
46	M	53	4 wk	17	33	45	234	150	64	0	0	10	Operation
47	F	52	6 wk	58	120	48	234	122	52	0	0	6	Operation
48	F	37	6 wk	39	68	57	270	124	45	0	1+	15	Operation
49	M	51	6 wk	23	39	59	260	156	60	0	0	15	Operation
50	M	74	8 wk	23	30	59	260	187	72	0	0	60	Operation
51	F	72	10 wk	11	21	52	262	141	54	0	Tr	14	Operation
52	F	50	12 wk	46	87	53	668	100	15	0	0	55	Operation
53	M	73	12 wk	14	30	47	112	73	65	0	1+	14	Operation
54	M	58	12 wk	31	65	48	346	197	57	0	1+	11	Operation
55	M	76	12 wk	16	34	47	252	108	43	0	0	14	Operation
56	F	44	4 mo	15	32	47	244	159	65	0	0	18	Operation
57	F	47	6 mo	53	112	47	794	193	49	0	0	33	Operation
58	F	52	6 mo	10	20	50	210	147	70	0	0	14	Operation
59	M	77	6 mo	51	99	52	158	55	40	2+	3	9	Operation
60	F	45	9 mo	44	70	03	336	185	55	0	0	19	Operation
61	F	72	1 yr	11	22	50	226	131	58	0	0	27	Operation
62	F	53	2 yr	10	22	45	294	218	74	0	0	18	Operation
63	M	74	2 1/2 yr	10	28	36	312	218	70	0	0	27	Operation
64	M	63	?	15	27	56	300	200	66	0	0	27	Operation
65	M	72	?	73	116	63	300	132	44	0	0	6	Operation
66	M	71	?	8	24	33	122	78	04	0	0	9	Operation
67	F	55	?	30	50	60	232	155	67	1+	1+	8	Operation

TABLE V RESULTS IN CASES OF EXTRAHEPATIC OBSTRUCTION DUE TO COMMON DUCT STRICTURE

CASE	SEX	AGE (yr.)	DURATION OF JAUNDICE	SERUM BILIRUBIN			CHOLESTEROL			CEPHALIN CHOLESTEROL		THYMOL TURBIDITY	ALANINE PHOSPHATASE	DIAGNOSIS ESTABLISHED BY
				1'	TOTAL	1'/T%	TOTAL	ESTER	ESTER%	24 HR.	48 HR.			
68	F	56	3 days	3.1	5.1	63	140	94	67	0	1+	3	34	Operation
69	M	66	7 wk	15.1	27.7	55	212	49	23	0	0	2	12	Operation
70	F	34	8 wk	3.5	6.8	51	362	188	52	0	0	2	37	Operation
71	M	36	12 wk	0.5	2.1	24	212	146	69	0	1+	1	20	Operation
72	M	31	12 wk	15.2	24.6	62	868	434	51	0	0	1	20	Operation
73	F	30	4 mo	2.1	4.1	51	294	150	51	0	0	3	23	Operation
74	F	67	1 yr	6.3	10.5	60	258	72	28	0	0	3	24	Operation
75	F	42	1 yr	5.5	10.2	54	920	488	53	1+	1+	6	50	Operation
76	F	49	7 yr	2.4	4.4	55	228	146	64	0	0	1	4	Operation
77	F	55	8 yr	4.2	6.9	61	380	213	56	0	0	1	20	Operation

TABLE VI. RESULTS IN CASES OF PARENCHYMAL LIVER DISEASE DUE TO CIRRHOSIS

CASE	SEX	AGE (yr.)	DURATION OF JAUNDICE	SERUM BILIRUBIN		CHOLESTEROL			CEPHALIN CHOLESTEROL		TILYMOL TURBID- ITY	ALKA LINE PHOS PHATASE	DIAGNOSIS ESTABLISHED BY
				1	TOTAL	1/T%	TOTAL	ESTER	ESTER%	24 HR	48 HR		
75	F	62	2 wk	138	218	63	276	19	7	4+	18	13	Biopsy
79	M	63	4 wk	64	100	64	124	53	43	0	3	3	Necropsy
80	M	30	4 wk	17	40	32	156	31	20	3+	4	3	Necropsy
81	M	70	6 wk	16	24	67	146	76	52	3+	7	2	Biopsy
82	M	42	10 wk	8	24	33	204	126	62	2+	24*	5	Biopsy
83	M	59	12 wk	21	39	54	170	76	45	3+	14	17	Necropsy
84	M	33	12 wk	131	276	47	96	22	23	4+	9	26	Biopsy
85	I	35	4 mo	24	51	47	384	230	60	2+	9*	14	Operation
86	F	62	5 mo	32	55	58	244	146	60	7+	10	14	Necropsy
87	M	54	6 mo	38	159	24	248	114	46	3+	21	11	Biopsy
88	F	12	9 mo	42	77	55	126	68	54	3+	19	13	Operation
89	M	32	11 mo	34	70	49	112	60	25	3+	3	3	Necropsy
90	M	30	1 yr	130	265	33	126	32	64	4+	2	3	Biopsy
91	M	58	1 yr	12	27	44	138	88	64	3+	9	59	Necropsy
92	F	44	2 yr	21	64	33	314	176	56	2+	6	18	Biopsy
93	F	42	3 yr	70	114	61	450	181	42	1+	7	18	Biopsy
94	F	51	3 yr	12	24	50	504	328	65	2+	16	13	Clinical data
95	F	17	3 yr	15	28	54	176	113	59	3+	6	12	Biopsy
96	F	20	3 yr	41	79	52	178	105	62	0	3	63	Operation
97	M	61	6 yr	15	35	43	248	154	57	0	1	28	Biopsy
98	F	37	7 yr	17	27	63	420	214	51	2+	21	4	Operation
99	F	54	10 yr	8	20	40	156	89	57	7+	10	6	Peritoneoscopy
100	M	8	?	10	32	31	108	55	51	4+	3	9	Biopsy
101	F	8	?	26	56	46	136	49	30	3+	10	19	Necropsy
102	F	10	?	13	20	45	92	30	33	4+	9	3	Biopsy
103	F	23	?	48	80	60	196	114	58	2+	3	3	Necropsy

By the method of Duclot.

TABLE VII RESULTS IN CASES OF PARENCHYMAL LIVER DISEASE DUE TO HEPATITIS

CASE	SEX	AGE (yr)	DURATION OF JAUNDICE	SERUM BILIRUBIN		CHOLESTEROL			CEPHALIN CHOLESTEROL		THYMOL TURBID ITY	ALKALINE PHOS- PHATASE	DIAGNOSIS ESTABLISHED BY
				I	TOTAL	1/T%	TOTAL	ESTER	ESTER%	24 HR	48 HR		
101	M	74	1 day	12.9	21.7	59	140	27	19	3+	3+	11	Biopsy
105	M	20	2 days	5.2	8.4	62	218	61	28	3+	4+	4	Clinical data
106	F	26	3 days	6.1	10.7	59	206	82	40	2+	3+	4	Clinical data
107	F	29	3 days	1.3	2.4	54	184	103	56	3+	4	8	Clinical data
108	M	56	3 days	2.9	5.6	52	230	135	58	0	0	8	Clinical data
109	M	50	3 days	4.6	7.7	60	170	85	50	3+	4+	3	Clinical data
110	M	12	4 days	5.7	9.2	62	153	23	15	2+	3+	9	Clinical data
111	M	59	4 days	25.0	39.0	64	176	42	24	3+	4+	8	Clinical data
112	M	26	4 days	3.8	7.5	51	242	145	60	2+	3+	10	Clinical data
113	F	19	6 days	3.1	6.0	47	210	65	31	3+	4+	4	Clinical data
114	M	33	7 days	7.8	13.0	60	166	61	37	3+	4+	8	Clinical data
115	M	64	7 days	15.0	27.7	54	226	34	15	3+	4+	7	Clinical data
116	M	4	8 days	4.3	7.3	59	244	44	18	2+	3+	13	Clinical data
117	M	20	8 days	1.7	4.6	59	132	61	46	2+	3+	5	Clinical data
118	F	20	9 days	2.8	5.4	52	196	59	30	1+	2+	6	Clinical data
119	F	32	10 days	1.8	3.0	60	252	88	35	3+	4+	8	Clinical data
120	F	19	10 days	1.9	3.5	54	236	109	38	3+	3+	8	Clinical data
121	F	20	11 days	3.2	7.0	46	186	93	49	3+	4+	7	Clinical data
122	M	31	14 days	11.7	19.0	62	154	31	20	3+	4+	4	Clinical data
123	F	34	3 wk	2.6	5.7	46	280	126	45	2+	2+	5	Clinical data
124	M	64	3 wk	3.0	5.2	78	490	314	64	3+	4+	15	Biopsy
125	F	12	3 wk	3.1	5.1	61	156	75	48	2+	2+	19	Clinical data
126	F	49	3 wk	1.2	7.5	56	214	66	31	3+	4+	12	Clinical data
127	F	46	3 wk	14.0	26.9	52	81	12	15	2+	3+	8	Necropsy
128	F	13	8 wk	2.6	4.8	54	100	30	30	2+	3+	30	Biopsy
129	M	11	8 wk	5.9	10.1	58	192	117	55	0	0	16	Clinical data
130	F	31	12 wk	7.7	15.2	51	118	61	52	3+	4+	3	Clinical data
131	M	32	4 mo	3.0	8.3	47	244	151	62	1+	1+	5	Biopsy
132	F	55	4 mo	9.2	16.1	57	262	94	9	+	+	9	Necropsy
133	F	18	6 mo	3.7	7.7	48	158	90	57	+	+	4	Clinical data
134	M	58	9 mo	9	2.1	43	260	163	65	1+	1+	8	Biopsy

\*By the method of Dacie<sup>12</sup>



TABLE VIII RESULTS IN CASES OF PARENCHYMAL LIVER DISEASE DUE TO MISCELLANEOUS CAUSES

CASE	SEX	AGE (YR.)	DURATION OF JAUNDICE	SERUM BILIRUBIN		1 / T %	CHOLESTEROL				CEPHALIN CHOLESTEROL		THYMOL TURBID ITY	ALBA LINE PHOS PHATASE	DIAGNOSIS ESTABLISHED BY
				1	TOTAL		TOTAL	ESTER	ESTER %	24 HR.	48 HR.				
135	F	39	4 wk	0.8	2.4	33	100	70	70	2+	3+	6	3	Necropsy	
136	M	57	4 wk	7.0	12.6	56	64	20	31	2+	3+	3	2	Necropsy	
137	M	71	8 wk	1.1	3.5	31	170	114	67	1+	2+	1	4	Clinical data	
138	M	45	9 mo	1.0	2.4	42	116	43	63	1+	1+	8	5	Clinical data	
139	M	52	?	1.7	2.7	63	206	144	70	2+	3+	3	3	Clinical data	
LYMPHOBLASTOMA															
140	F	15	2 wk	2.9	5.0	51	90	8	9	1+	2+	2	1	Necropsy	
141	M	47	7 wk	0.7	2.9	24	370	74	20	0	Tr	2	10	Biopsy	
SEPTICEMIA															
142	M	1	7 wk	3	7.4	40	126	59	47	Tr	1+	2	18	Blood culture	
143	M	70	?	4.3	6.0	65	90	42	47	1+	2+	1	12	Blood culture	
SULFA REACTION															
144	M	13	6 days	7.5	13.1	57	200	44	22	1+	1+	6	15	Necropsy	
MYELODYSPLASIA															
145	M	68	?	0.6	2.4	33	226	131	53	0	0	10	4	Necropsy	
PERNICIOUS ANEMIA															
146	M	73	6 mo	0.6	2.5	24	152	96	63	0	0	1	1	Hematology	
FATTY METAMORPHOSIS															
147	F	26	?	4.8	8.7	50	34	4	12	1+	2+	1	7	Necropsy	

# Quantitative Serum Bilirubin

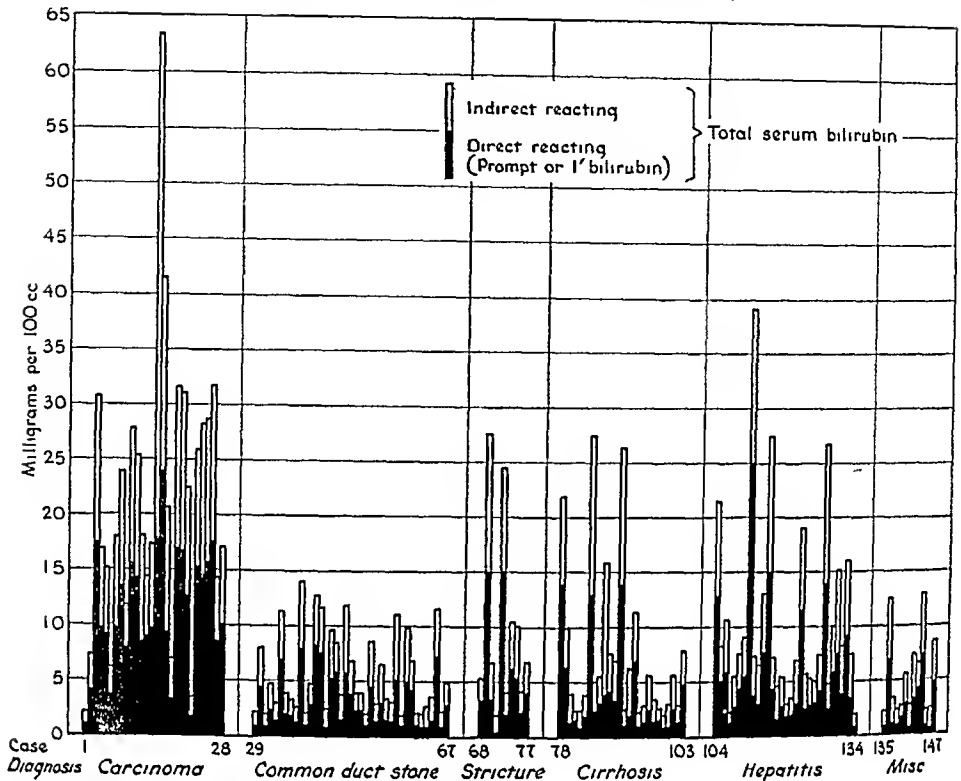


Fig 1

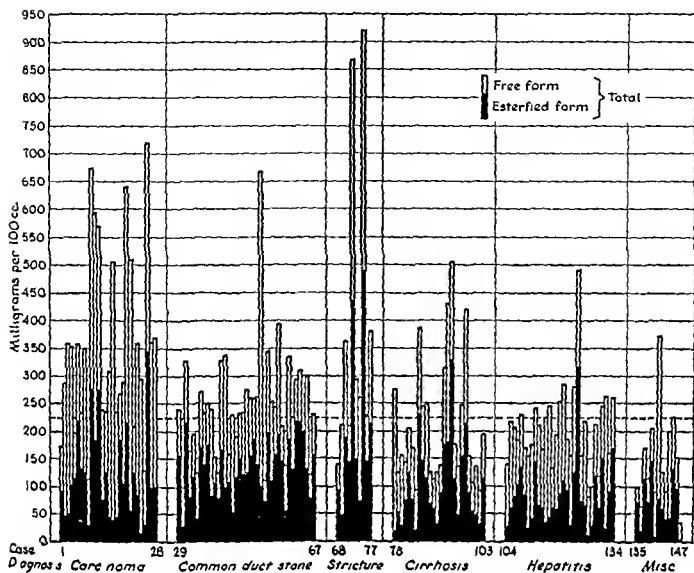
The cholesterol data are arranged in Fig 2 in the order of the duration of jaundice

*Serum Alkaline Phosphatase*—The results of the individual tests appear in Tables III through VIII. The instances in which the anticipated values were not obtained are listed in Table IX. It may be seen that the results were quite similar to those obtained with the cholesterol determination. The data are presented graphically in Fig 3 in the order of the duration of jaundice.

TABLE IX NUMBER OF INSTANCES IN WHICH ACTUAL RESULTS FAILED TO CONFORM WITH ANTICIPATED RESULTS

TYPE OF DISEASE	NUMBER OF CASES	CEPHALIN CHOLESTEROL	THYMOL TURBIDITY	SERUM CHOLESTEROL	CHOLESTEROL ESTER (PLP CENTAGE)	ALKALINE PHOSPHATASE
Carcinoma	28	2	4	5	21	3
Common duct stone	39	2	1	12	11	12
Common duct stricture	10	0	1	3	2	1
Total	77	4	6	20	34	16
Cirrhosis	26	9	9	9	16	14
Hepatitis	31	5	6	11	10	8
Miscellaneous hepatic disease	13	10	9	2	6	1
Total	70	24	21	22	32	20

## Serum Cholesterol



Fig

## Alkaline Phosphatase

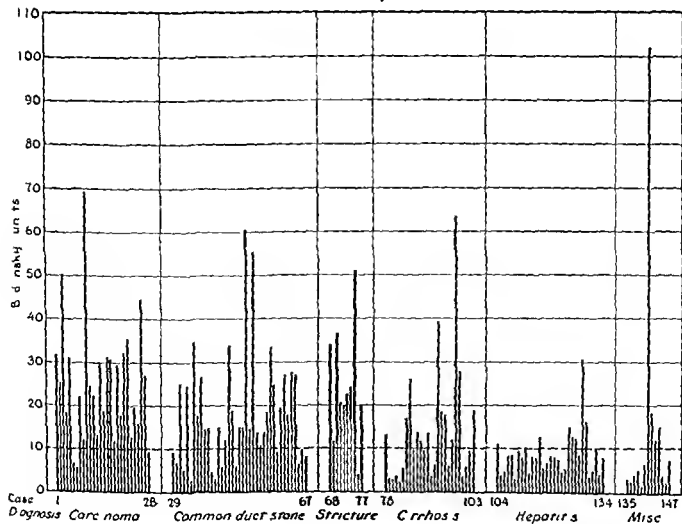


Fig 3

*Cephalin-Cholesterol Flocculation Test*—For the purpose of this analysis only the reading obtained at twenty-four hours has been considered although both values are presented in Tables III through VIII. The data for the twenty-four hour reading have been arranged according to the duration of

### Cephalin Cholesterol Flocculation at 24 hours

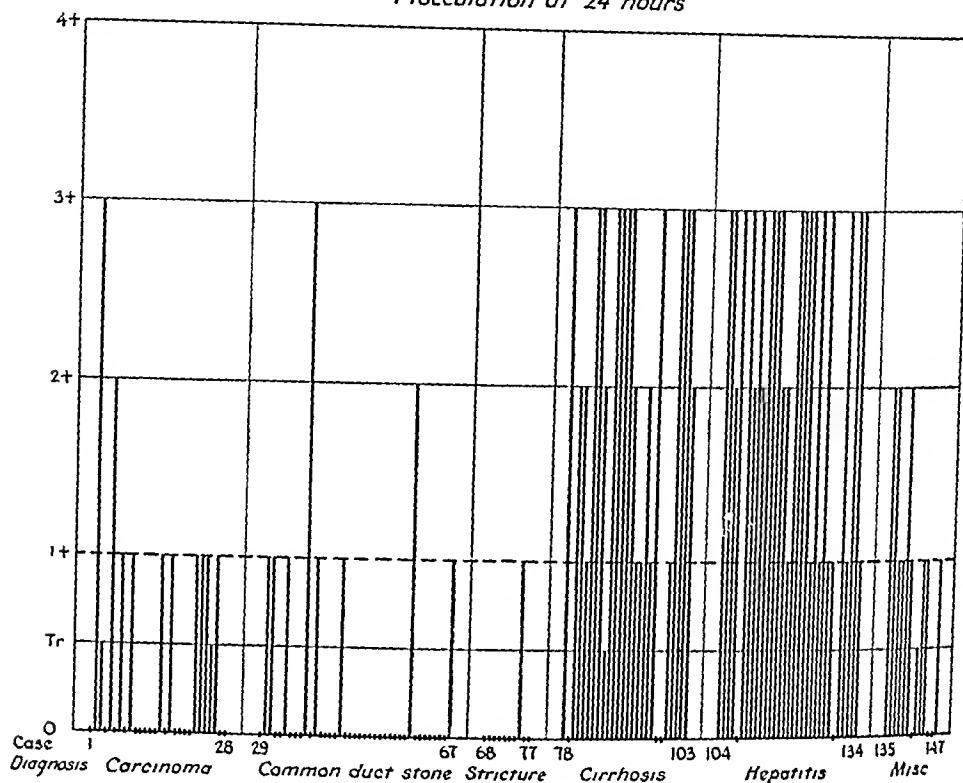


Fig 4

jaundice in Fig 4. Among the seventy-seven cases of extrahepatic biliary obstruction there were four instances in which abnormal, and therefore presumably misleading, results were obtained.

The two patients in the carcinomatous obstructive group (Cases 2 and 5, Table III) both exhibited positive Hanger tests early in the course of their jaundice. In Case 2, the patient, an 83-year-old woman, had jaundice due to a carcinoma of the gall bladder. Subsequent cephalin cholesterol flocculation tests also were positive repeatedly. The patient of Case 5, a 56-year old man, proved to have a carcinoma of the pancreas with hepatic metastases. Serial determinations were not possible in this instance.

There were two proven instances of common duct stone obstruction in which the Hanger test was positive (Cases 38 and 50, Table IV). Jaundice associated with chills and fever had been present for seven days in Case 38, one week later the cephalin cholesterol was only a trace at 24 hours, and though

jaundice had diminished, the other tests remained essentially unchanged. The presence of a positive test in Case 59 was not surprising since the patient had been jaundiced for six months.

There were twenty four cases among the seventy examples of parenchymal liver disease in which the Hanger test was negative. This should occasion no surprise since the group is a heterogeneous one, and save for the thirty one cases of hepatitis, uniform results would be unlikely.

### Thymol Turbidity Reaction

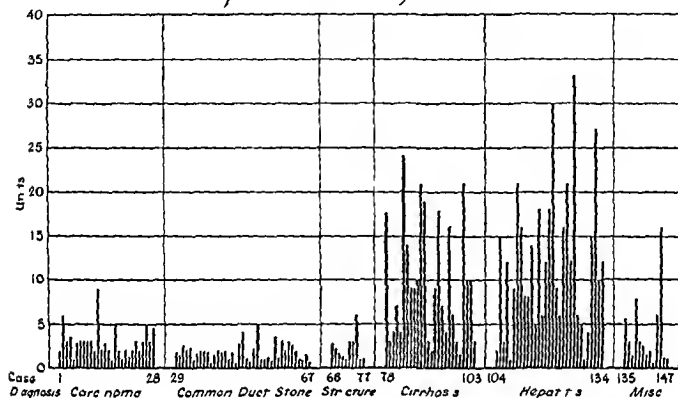


Fig 5

**Thymol Turbidity**—The data relating to the thymol turbidity test obtained from Tables III through VIII, are arranged graphically in Fig 5 according to duration of jaundice. Inspection of Table IX and Fig 5 reveals that the test gave positive results (values greater than 4 units) in four cases of carcinoma and one each in the patients with calculus or stricture.

Among the cases of cancerous jaundice with abnormal thymol turbidity tests three patients (Cases 2, 17 and 26) had values just slightly above the level of 4 units which is taken as the dividing line. Case 12 revealed a value of 9 units. Only one patient in the group of calculus jaundice had a value above 4 units. This was Case 52 with 5 units. Similarly one patient (Case 75 Table V) with a long standing stricture of the bile duct revealed a thymol test of 6 units.

The seventy cases of parenchymal liver disease included twenty four instances in which the thymol turbidity test was not elevated (Table IX).

**Multiple Tests**—The principal purpose of this study was to determine the merit of multiple laboratory tests that can be performed on a single sample of serum. It is therefore necessary to analyze briefly how often certain combinations of these procedures gave misleading information. It is evident that the

quantitative van den Bergh reaction (Fig 1) failed to give information of differential diagnostic value in this series of cases of regurgitation jaundice. The serum bilirubin will not, therefore, be considered further in relation to any combination.

The ester fraction of the serum cholesterol, when expressed as per cent of the total, failed to conform to the anticipated results in thirty-four cases in the extrahepatic obstructive group. In thirty-two cases in the parenchymal jaundice group the value of the cholesterol ester fraction was greater than 50 per cent and may, therefore, be considered unexpected. Because there appeared to be so much overlapping of values irrespective of what percentage level was chosen, this particular determination also will be eliminated from the present discussion of multiple tests.

Four procedures—the total cholesterol, the alkaline phosphatase, the cephalin-cholesterol flocculation, and the thymol turbidity—remain then for comparison. This comparison can best be made by examining the cases according to the established diagnoses.

*A Carcinoma* Results opposite to those anticipated were encountered in two cases, two of the four tests proved to be misleading. Case 2, in which both the cephalin-cholesterol and the thymol test were positive, has been discussed previously. The results were consistent on repeated examinations during the forty-five days that the patient survived. This was the only case in the group of seventy-seven surgical patients in which both the cephalin cholesterol and the thymol turbidity were simultaneously positive and thereby misleading.

Case 5 also has been discussed previously. In that instance the cephalin cholesterol flocculation value at 24 hours was 2 plus and the total cholesterol was only 198 mg per 100 cubic centimeters. Only one set of determinations was made in this patient.

*B Common Duct Stone* In one patient (Case 59) in this group, three of the four tests under discussion were misleading. This elderly man had been jaundiced for six months. The total cholesterol was only 138 mg per 100 cc, the cephalin-cholesterol reading was 2 plus, and the alkaline phosphatase value was 9 Bodansky units. Obstruction to bile outflow was presumably high grade since the total serum bilirubin at this time was 9.9 mg per 100 cubic centimeters. The fecal urobilinogen was not determined, but the presence of increased urobilinogen in the urine indicated incomplete obstruction.

In six cases in this group, two of the four tests failed to conform to the expected results. In each of these six instances (Cases 30, 32, 34, 40, 43, and 66) the total cholesterol ranged from 100 to 164 mg per 100 cc and the alkaline phosphatase was not elevated above 10 Bodansky units. The negative flocculation and turbidity tests in these instances were thus most helpful from the differential diagnostic standpoint.

*C Stricture* In no instance in these ten cases was more than one of the four tests incorrect in any one patient.

*D Cirrhosis* The results listed in Table IX indicate that the individual tests were frequently misleading in the twenty-six cases of cirrhosis. In Case

98 all four of the procedures under discussion yielded results that were misleading as judged by the arbitrary standards. This patient did have a cirrhosis as proved by the surgical biopsy specimen. The patency of the bile duct was established by cholangiography at the time of surgery. The patient was believed to represent an example of cholangiolitic cirrhosis<sup>14</sup>. Further evidence of the regurgitation of bile was the elevated blood bile acid patient, 38, control 0.7 mg per 100 cc as determined by the method of Josephson<sup>15</sup>.

Four cases (Cases 93, 95, 97 and 99) gave results that were misleading with three of the four tests. These results were particularly disturbing in Cases 95 and 99 because both the thymol turbidity and the cephalin cholesterol tests gave normal values and the phosphatase levels were high. Both patients were middle aged women with jaundice of long standing and cirrhosis of unknown etiology. The patency of the bile ducts was established in each instance by subsequent exploratory operation. The cirrhotic process was not due to biliary stricture or ascending cholangitis so far as could be determined. In the remaining two instances (Cases 93 and 97) the thymol turbidity alone behaved in the expected fashion. Necropsy performed in Case 93 revealed a finely nodular 2625 gram liver that was classified as a portal cirrhosis of the Laennec type by the pathologist. The bile ducts were patent; the gall bladder had been previously removed surgically. In Case 97 the patient had had jaundice and pruritus of six years. A biopsy diagnosis of cirrhosis had been made previously and the patency of the bile duct established by operation. Although the cholesterol and phosphatase values were not remarkably elevated the blood bile salt level was (patient 4.1 control 0.7 mg per 100 cc). Clinically this patient also was believed to represent an instance of cholangiolitic cirrhosis.

In ten cases in the cirrhotic group two of the four tests proved to be misleading. In two of these Cases 79 and 82 this was especially serious in that both the thymol turbidity and the cephalin cholesterol tests gave negative results. Thus biochemically they closely resembled the previously mentioned six cases of common duct stone that failed to show the expected elevations of cholesterol and phosphatase. The other eight cases were characterized mainly by misleading elevations of the cholesterol or phosphatase (Table VI).

*E Hepatitis* The figures listed in Table IX show considerable deviation from the anticipated results in the thirty one cases of hepatitis. The results are slightly superior to those obtained in cirrhosis however possibly because this is a more homogeneous group. The criticism may be made that we have employed certain biochemical tests to establish a diagnosis of hepatitis and then have proceeded to use this diagnosis to demonstrate the value of the tests. This weakness in such a study is appreciated but in the absence of any specific biologic tests for virus hepatitis all such diagnoses in the absence of a biopsy must be based on clinical features and course.

In two cases in this group the results of these four tests were quite misleading in that three of the four procedures gave results more in keeping with the arbitrary standards listed for extrahepatic obstruction. Case 108 (Table VII) failed at any time to show a positive result by either the Hauger or the

Maclagan tests and the initial cholesterol level was 230, serial observations showed a rise to 398 mg per 100 cc and an eventual decline to 225. Yet, the clinical course appeared to leave little doubt that the jaundice was due to hepatitis. Complete recovery ensued after a three-week illness, subsequent examination by cholecystography revealed a normally functioning gall bladder. Case 129 (Table VII) was very similar in regard to these tests. The patient was followed by serial tests at intervals of one week. The tests performed during the fifth and sixth weeks revealed total serum bilirubin levels of 2.6 and 1.5 mg per 100 cc respectively. On these two occasions only, the cephalin-cholesterol test was 2 plus and the thymol test 6 units. Subsequent values were again normal and complete clinical recovery ensued.

In six of the cases of hepatitis, two of the four procedures gave misleading results. In three of these (Cases 112, 116, and 124) the cholesterol and the phosphatase were elevated rather than normal or decreased as might be expected in hepatitis, the abnormal turbidity and flocculation tests were therefore of considerable diagnostic value in these two instances. Cases 131 and 134 failed to demonstrate cephalin-cholesterol values greater than 1 plus at 24 hours and the total cholesterol values were higher than might be anticipated in hepatitis. The thymol turbidity value was only 2 units and the alkaline phosphatase level was 11 Bodansky units in Case 104.

*F Miscellaneous Hepatic Disease* This small group of thirteen patients has been included in this study chiefly because they had sufficient elevation of the serum bilirubin to conform with the criteria previously listed and because the tests under consideration were performed simultaneously. In general, the clinical nature of their illnesses was such that tests of liver function were not employed particularly for differential diagnosis. If judged arbitrarily, however, the results of the tests were misleading in most instances. The serum cholesterol showed to best advantage in this group. The low value in Case 147 was particularly striking. At necropsy, this 27-year-old woman was found to have a markedly fatty liver as a complication of severe and intractable ulcerative colitis of three months' duration.

#### DISCUSSION

The purpose of this study was to evaluate the merits of what has been termed a "minimal yet adequate" program of liver function studies in the differential diagnosis of jaundice.<sup>4</sup> For this analysis, the program has been minimal since only the initial single set of determinations obtained in each patient has been evaluated. The limitations and merits of the individual tests have been so frequently and thoroughly discussed in current literature that no attempt will be made to review that aspect here. The group of tests employed in this study can be performed on a single sample of serum and technically represent procedures that are well within the range of a modern hospital laboratory. The merits of these procedures in differential diagnosis have been subjected to critical testing on the basis of rather arbitrary standards.



The quantitative van den Bergh reaction when used as a means of determining the bilirubin content of the serum has much to recommend it. The very real clinical value of the test in differentiating retention and regurgitation jaundice has been mentioned. Malloy and Evelyn<sup>10</sup> indicated that the quantitative van den Bergh did yield results that would permit the differentiation of the various forms of regurgitation jaundice, a view shared by Meakins.<sup>17</sup> Ducci<sup>13</sup> indicated on the basis of his extensive experience in 1,770 cases, that the percentage of the prompt direct reacting bilirubin bore some relationship to the type of jaundice. In hepatocellular jaundice he observed the value to be "over 50 per cent," in incomplete obstructive posthepatic jaundice, "generally over 60 per cent," and in complete obstructive posthepatic jaundice, "generally over 70 per cent." In the present small series of cases the relationship of the degree of biliary obstruction to the behavior of the serum bilirubin has not been studied. It is apparent from Fig. 1 that the levels of the one minute and the total bilirubin and the percentage relationship were too variable to have diagnostic significance.

The serum cholesterol levels and particularly the level of the cholesterol ester in the jaundiced patient have long been employed to good advantage. We have been repeatedly impressed with the merit of serial determinations of the total and esterified cholesterol in evaluating the severity of liver damage. The occurrence of low values for the total serum cholesterol in jaundice usually indicates severe hepatocellular dysfunction. This is particularly true when there is an associated sharp reduction in the concentration of the esterified fraction.<sup>18, 21</sup> The results obtained in the twenty eight cases of jaundice due to cancer indicate that the cholesterol ester fraction did not increase to the same degree as did the free form. In four of the cases (Table III) the total cholesterol was greater than 225 mg per 100 cc and the ester fraction comprised more than 50 per cent. In nineteen of the cases, though the total cholesterol exceeded 225 mg per 100 cc, the ester fraction was below 50 per cent. In nine of these nineteen cases the absolute value for the ester was below 100 mg per 100 cubic centimeters.

In the instances of calculus jaundice (Table IV) twenty of the thirty nine patients exhibited total cholesterol values greater than 225 mg per 100 cc and esterified fractions greater than 50 per cent. Seven of these had total cholesterol values greater than 225 mg per 100 cc with less than 50 per cent esterified. However in only two of these cases was the absolute value for the ester below 100 mg per 100 cubic centimeters.

On the basis of this limited number of cases it might be suggested that this difference of behavior of the serum cholesterol ester in cancerous and calculus jaundice has some diagnostic significance. It should be noted that Epstein and Greenspan<sup>19</sup> found elevated cholesterol ester levels as well as elevated total cholesterol levels in 75 per cent of their fifty two cases of jaundice due to cancer, a finding at variance with earlier reports.<sup>21</sup> Reduced serum cholesterol ester percentages in jaundice due to cancer have been recorded by others.<sup>3</sup> This

and other aspects of the serum cholesterol behavior in jaundice do not warrant undue emphasis, however. Snell<sup>24</sup> has indicated the difficulties encountered in attempting such correlations.

The variations of the serum alkaline phosphatase activity in jaundice have been utilized for diagnostic purposes since 1933.<sup>25, 26</sup> Gutman and associates<sup>27</sup> reported that the majority (approximately 90 per cent) of cases of extrahepatic biliary obstruction had levels of alkaline phosphatase activity greater than 10 Bodansky units. In many cases of diffuse liver damage, the alkaline phosphatase failed to exceed 10 Bodansky units. The diagnostic merits of combining the phosphatase determination with the cephalin-cholesterol flocculation test<sup>28</sup> or with the thymol turbidity test<sup>29</sup> have been described. The results of the present study are in agreement with the observations<sup>27</sup> that there is better correlation of elevated alkaline phosphatase in jaundice due to cancer than in that due to calculus obstruction (Table IX). The fact that certain cases of diffuse liver damage have phosphatase values greater than 10 Bodansky units is also well recognized.<sup>27</sup> The results shown in Table IX and in Fig. 3 bear this out.

The present study indicates that the cephalin-cholesterol and thymol turbidity tests, when considered together, were seldom misleading in the surgical cases. In only one instance in the seventy-seven cases of extrahepatic obstruction (Case 2) were both tests simultaneously positive on this single examination. The opposite aspect, that is the failure of the tests to exhibit positive reactions in the presence of parenchymal liver disorders, is more common. The two instances in the hepatitis group in which both tests were negative (Cases 108 and 129) have received comment. There were five such instances in the cirrhotic group and seven in the miscellaneous group.

It seems apparent from recent observations that the zinc turbidity test of Kunkel<sup>30</sup> yields fewer false positive reactions in cases of extrahepatic biliary obstruction than does the thymol turbidity. As Popper and associates<sup>31</sup> have observed, the zinc turbidity test will likely prove to be of additional aid in the laboratory diagnosis of jaundice.

In summary, it may be stated that certain characteristic biochemical responses do occur fairly uniformly as the result of hepatic disease and bile duct obstruction. Most cases of extrahepatic bile duct obstruction exhibit elevated cholesterol and alkaline phosphatase levels and have normal responses to the thymol turbidity and the cephalin-cholesterol flocculation tests. Many cases of jaundice due to diffuse liver disease exhibit biochemical changes of an opposite nature, consequently such tests have differential diagnostic value. Certain cases of parenchymal liver disease exhibit changes that closely parallel those seen in extrahepatic obstruction, i.e. elevated cholesterol and phosphatase values and normal responses to flocculation and turbidity reactions. This constitutes a limitation of the use of so called tests of liver function in differential diagnosis that must be accepted. It is a reflection of a basic pathologic phenomenon—the diffuse liver damage existing in hepatitis and cirrhosis mix-

manifest itself with varying emphasis on hepatocellular or cholangiolar functional impairment. Hepatocellular functional impairment may be regarded as the classical type and the resultant biochemical changes are quite characteristic and readily recognizable. When cholangiolar functional impairment predominates in cases of hepatitis and cirrhosis, the results of the tests under discussion may prove misleading insofar as differential diagnosis is concerned.

#### CONCLUSIONS

1 The results of five biochemical procedures (bilirubin, cholesterol, phosphatase, cephalin cholesterol flocculation and thymol turbidity) applicable to a single sample of serum have been analyzed as to their respective merit in the differential diagnosis of jaundice.

2 The results in seventy seven patients with jaundice due to extrahepatic biliary obstruction have been contrasted with those obtained in seventy patients with jaundice due to parenchymal liver disease.

3 The results of these tests in jaundice due to extrahepatic obstruction were fairly uniform, nevertheless deviation from the anticipated results in two or more of the tests occurred in ten instances.

4 The results of these tests in jaundice due to parenchymal liver disease were variable. They were adequate from a diagnostic standpoint in the cases where hepatocellular dysfunction predominated. In a significant number of cases, cholangiolar dysfunction apparently predominated and the results of the tests resembled those seen in extrahepatic biliary obstruction.

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# EFFECT OF EPINEPHRINE ON VITAMIN A AND GLUCOSE BLOOD LEVELS IN NORMAL AND CIRRHOTIC SUBJECTS

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THE role of the liver in the regulation of the blood plasma vitamin A level is poorly understood. Although accorded diagnostic significance in hepatic disorders, the plasma concentration does not necessarily reflect the vitamin A concentration in liver tissue.<sup>1-9</sup> On diets restricted in vitamin A the blood content ordinarily is maintained within the normal range virtually to the point of full tissue depletion. Moreover although 95 per cent of the body's vitamin A is stored in the liver,<sup>10</sup> the recent work of Sexton and co-workers<sup>11</sup> suggests that this organ may not be, as was hitherto generally believed, the principal site of conversion from the carotene precursor.

Hepatic diseases, however are often associated with lowered blood vitamin A levels.<sup>12-16</sup> This occurs in cirrhosis in which the vitamin A content of the liver is greatly reduced.<sup>15-17</sup> The low plasma levels in cirrhosis might reflect an inability to mobilize the vitamin because of decreased body stores.

Young and Wald<sup>18</sup> noted an increase in the blood vitamin A level of rabbits following epinephrine injection. Observations made in this laboratory indicated that the same phenomenon often occurs in normal human subjects.\* This phenomenon was utilized in the present investigation to study the mobilization of vitamin A in patients with cirrhosis. The blood glucose response to epinephrine, which is thought to be a measure of hepatic glycogenolysis<sup>10-19</sup> and may be otherwise related to vitamin A metabolism,<sup>5, 6, 10, 20, 21, 22</sup> was determined simultaneously.

## MATERIAL AND METHODS

Studies were made in twelve patients with cirrhosis of the liver and twenty-two control subjects, in two of whom the procedure was repeated. Two of the twelve cirrhotic subjects had complicating diabetes mellitus and one (with previous jaundice but no clinical diabetes) was discovered at autopsy to have hemochromatosis. The control group—recruited from non-cirrhotic patients, staff members and medical students—included ten persons with completely normal liver function studies and twelve without clinical evidence of hepatic disease but with one or more often transitory, abnormal function tests. One of each of these groups was diabetic.

The patients with cirrhosis were on a standard high protein, high calorie diet.<sup>23</sup> Except for appropriate diabetic regimens in two cases there was no regulation of food intake among the control subjects.

The test was performed in the fasting state and consisted of the intramuscular injection of epinephrine (0.6 cc. of a 1:1000 solution). Samples of venous blood were taken before and at one-half, one and two hour intervals after the injection. Plasma vitamin A (and carotene) levels were determined by the method of Kimble<sup>24</sup> employing the Carr-Price reaction. Blood sugar concentrations were measured by a modified Benedict procedure.<sup>2</sup>

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The reciprocal phenomenon, i.e. a decrease in the plasma vitamin A level likewise has been observed following injection of an epinephrine antagonist, insulin and, on occasion following the injection of glucose alone.

\*Simultaneous determinations have shown essentially parallel changes in arterial and venous blood plasma vitamin A levels.

## RESULTS

Results are shown in Table I

Following administration of epinephrine, the maximum variation in the direction of initial change is recorded in terms of percentage of the preinjection level of each constituent measured. In the case of vitamin A an increase of 5 per cent or more is arbitrarily regarded as significant, i.e. a positive test. A

TABLE I. BLOOD VITAMIN A, CAROTENE, AND SUGAR LEVELS FOLLOWING THE INTRAMUSCULAR INJECTION OF EPINEPHRINE IN PATIENTS WITH CIRRHOSIS OF THE LIVER (WITH MEAN VALUES FOR THE CIRRHOSIS AND CONTROL GROUPS)

PATIENT	BLOOD SUBSTANCE	BLOOD LEVEL SPECIMEN				PER CENT INITIAL CHANGE
		FASTING	½ HR.	1 HR.	2 HR.	
1 (C)	Vitamin A	57	52	50	45	-21
	Carotene	389	385	395	356	- 1
	Sugar	77	80	79	84	+ 9
2	Vitamin A	44	57	59	40	+34
	Carotene	115	115	112	108	- 6
	Sugar	97	103	104	102	+ 7
3 (D)	Vitamin A	46	60	48	47	+30
	Carotene	158	162	166	158	+ 5
	Sugar	117	129	142	153	+31
4 (C)	Vitamin A	86	82	81	87	- 6
	Carotene	279	303	303	293	+ 9
	Sugar	78	107	99	103	+37
5	Vitamin A	93	93	99	85	- 9
	Carotene	117	108	115	105	- 8
	Sugar	78	93	111	90	+42
6	Vitamin A	114	117	110	120	+ 3
	Carotene	92	97	72	96	+ 8
	Sugar	65	99	103	81	+58
7	Vitamin A	100	109	102	94	+ 7
	Carotene	99	110	107	99	+11
	Sugar	88	99	103	82	+17
8 (C)	Vitamin A	107	128	114	114	+20
	Carotene	178	187	128	188	+ 5
	Sugar	90	90	98	97	+23
9 (D)	Vitamin A	124	92	91	90	-26
	Carotene	100	96	99	99	- 4
	Sugar	124	218	195	169	+75
10	Vitamin A	32	42	37	48	+50
	Carotene	139	150	100	146	+ 7
	Sugar	65	70	74	75	+15
11	Vitamin A	38	32	31	29	-24
	Carotene	69	69	77	68	+12
	Sugar	80	92	90	90	+15
12	Vitamin A	28	27	20	31	-29
	Carotene	73	74	75	73	+ 3
	Sugar	64	70	76	73	+19
Cirrhosis group	Vitamin A		Mean	12 Tests		Range
	Carotene					+ 2 (-29 to + 50)
	Sugar					+ 3 (- 8 to + 12)
Control group	Vitamin A		Mean	24 Tests		+29 (+ 7 to + 58)
	Carotene					+ 3 (-17 to + 24)
	Sugar					0 (-17 to + 15)
						+17 (+ 4 to +156)

(C) Patient receiving carotene

(D) Patient with diabetes mellitus

blood sugar elevation of 30 per cent above the fasting figure is regarded as normal—virtually the same value as that generally accepted for the response to epinephrine<sup>10 13</sup>

In five of twelve (42 per cent) determinations in the cirrhotic group and in ten of twenty four (42 per cent) in the control group there was a significant increase in plasma vitamin A after injection of epinephrine. Among the twelve patients with cirrhosis only one showed a significant increase in both plasma vitamin A and blood sugar levels. The vitamin A level alone rose in four others, while a normal blood sugar elevation occurred in four without a positive vitamin A change. Three individuals showed no significant positive shift in either constituent.

Of the control subjects eight yielded a rise in both blood vitamin A and sugar levels, two an elevation in the vitamin A alone, eleven, in the sugar alone, and three, in neither substance.

Six patients with cirrhosis and seven of the control group showed decreased vitamin A levels after injection of epinephrine.

Blood plasma carotene levels measured as a necessary part of the Kimble procedure, showed no consistent changes in either group and served as a check on possible errors due to faulty sampling or variations in blood volume.

There was no apparent correlation between the initial blood concentration and the magnitude of the individual response. However, the most pronounced reductions in vitamin A levels were exhibited among individuals with low fasting levels.

Of the two control subjects in whom the test was performed twice one exhibited a rise in vitamin A and blood sugar on both occasions and the other showed a vitamin A rise in one and a blood sugar increase in both instances.

#### DISCUSSION

The plasma vitamin A response to the injection of epinephrine is apparently a variable one. While approximately 42 per cent of individuals exhibit an arbitrarily significant increase in plasma vitamin A concentration, the majority show a trend in that direction and only a few undergo a decrease of comparable magnitude. Since an elevation of plasma vitamin A level occurs with virtually the same frequency among patients with cirrhosis as among normal controls the response to injected epinephrine in the dose employed cannot be used as a test of hepatic function. Moreover the frequent failure of control subjects to demonstrate the so called normal mobilization of blood sugar following injection of epinephrine<sup>10 13</sup> casts doubt on the validity of this test as well. This finding is at variance with the opinion recently expressed by Kinsell and associates<sup>24</sup>

Probably most significant is the demonstration that blood plasma vitamin A levels evidently are not a reliable index of body stores since individuals with abnormally low levels may show an increase in the concentration of this substance in response to injections of epinephrine.

## SUMMARY

Plasma vitamin A and blood sugar responses to injection of epinephrine were determined in twelve patients with cirrhosis of the liver and in twenty two control subjects

A significant increase in plasma vitamin A concentration was observed in 42 per cent of each group. There was no apparent correlation between initial levels and the magnitude of the vitamin A responses. Low fasting concentrations did not preclude a significant increase in plasma vitamin A. There was no correlation between the responses of blood vitamin A and blood sugar to epinephrine administration.

The plasma vitamin A response to epinephrine injection does not appear to be a valid test of hepatic function.

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# INTESTINAL PARASITISM IN AMERICAN TROOPS IN GERMANY

## RELATION TO TRANSMISSION OF VIRAL HEPATITIS

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THE epidemiology of the sporadic, endemic hepatitis now occurring in Germany among American Occupation Troops is not clear, and it is impossible, in the absence of precise laboratory methods, to distinguish clinically homologous serum jaundice (SH) ‡ from infectious hepatitis (IH). As the manner of spread of the two infectious agents is different, with consequent difference in methods of prevention and control, it is important to determine which agent is the predominant one in Germany.

It has been shown definitely that the oral-intestinal route may be used to infect human volunteers with the agent of infectious hepatitis (virus IH), by Voegt,<sup>2</sup> by MacCallum and Bradley,<sup>3</sup> and by Havens and co-workers.<sup>4</sup> Whether there are other routes whereby the disease could spread naturally is not known. On the other hand, the virus SH of "homologous serum hepatitis" has not been transmitted by the oral route—with two possible exceptions<sup>6, 7</sup> which American workers<sup>8, 9, 10, 11</sup> have been unable to confirm. It was thought that at least a partial idea of the amount of fecal contamination in the food of soldiers with hepatitis might be gained by surveying the amount of intestinal parasitism present among them and by comparing the findings with those of soldiers in other Army hospitals who did *not* have hepatitis, with presumably healthy soldiers, and with the indigenous German personnel employed in Army messes.

Although as an oblique study of one facet of the epidemiological problem involved, no significant information was acquired, the findings of widespread intestinal parasitism in American troops in Germany seems worth reporting to call attention to the high incidence of parasitism in all groups studied.

## MATERIALS AND METHODS

At the Hepatitis Research Center, 220 patients with hepatitis were examined. The patients were in all stages of the disease, from the acute period to convalescence. The diagnosis of hepatitis had been established by the history, the appearance of icterus, and liver function tests. Patients with carbon tetrachloride poisoning with icterus were excluded from the series, as were patients in whom the diagnosis was doubtful or in whom the icterus had been determined to result from cholelithiasis.

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A preliminary report of some of this work has appeared in the Medical Bulletin EUCOM.<sup>12</sup>

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‡The terms SH and IH have been suggested by Neefe<sup>1</sup> to designate homologous serum jaundice and infectious hepatitis respectively

As a comparable hospital population, the EUCOM Orthopedic Center (to which, as to the Hepatitis Center, patients are sent from all parts of Germany and Austria) was selected, and 116 orthopedic patients without hepatitis were examined. In addition, a survey of 138 patients on all services in the 279th Station Hospital in Berlin (excluding those with hepatitis awaiting shipment to the Hepatitis Center) was made. This hospital was selected because it receives patients chiefly from the Berlin area where the rate of hepatitis is somewhat higher than in the remainder of Germany and is, furthermore, not in a highly endemic area for any particular parasite while the Orthopedic Center was at Stuttgart where there is a high incidence of ascariasis among the indigenous population. The incidence of intestinal protozoa is quite uniform throughout the American Zone of Occupation. In addition, 190 presumably healthy soldiers from the hospital in Berlin were examined. At the same time parasitological examinations on the stools of 213 indigenous personnel\* employed in the hospitals were made, results of which have been combined with the findings on previous surveys (July, 1947, to April 1948) of 1492 indigenous mess personnel from various Army installations all over Germany. Altogether a total of 644† military† and 1705 indigenous personnel are herewith reported. The composition of the groups examined and the findings are given in Table I.

TABLE I INCIDENCE OF PARASITISM IN AMERICAN AND INDIGENOUS PERSONNEL IN THE AMERICAN ZONE OF OCCUPATION GERMANY

GROUPS SURVEYED	1	2	3	4	TOTALS OF GROUPS 1, 2, 3, 4	5
DATE OF SURVEY	NOV 1947	APR 1948	JAN 1948	AIR. 1948		JUL 1946 APR 1948
NUMBER OF PERSONS	212	111	134	187	644	1705
Endamoeba histolytica	35 (16%)	12 (11%)	13 (10%)	14 (7%)	74 (11%)	2%
Endamoeba coli	38 (18%)	32 (29%)	27 (20%)	43 (23%)	140 (22%)	44%
Endolimax nana	81 (38%)	49 (44%)	32 (24%)	39 (21%)	201 (31%)	37%
Iodamoeba buetschlii	21 (10%)	4 (4%)	6 (4%)	8 (4%)	39 (6%)	11%
Dientamoeba fragilis	3 (1%)	9 (8%)	0 (0%)	10 (5%)	22 (3%)	5%
Giardia lamblia	16 (8%)	15 (14%)	14 (10%)	12 (6%)	57 (9%)	8%
Chilomastix mesnili	6 (3%)	2 (2%)	3 (2%)	1 (0.5%)	12 (2%)	3%
Ascaris lumbricoides	2 (1%)	2 (2%)	1 (1%)	2 (1%)	7 (1%)	10%
Trichocephalus trichiura	2 (1%)	2 (2%)	2 (1%)	0 (0%)	6 (1%)	11%
Enterobius vermicularis	4 (2%)	1 (1%)	3 (2%)	0 (0%)	8 (1%)	3%
Hymenolepis nana	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (0.1%)	0.1%
Hookworm	10 (5%)	8 (7%)	0 (4%)	10 (5%)	34 (5%)	0.1%
Strongyloides stercoralis	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (0.1%)	0.1%
Number of infestations	218	138	107	130	602	2678
Infestations† per 1,000 persons	1028	1243	798	743	935	1571
Persons harboring parasites	128 (60%)	71 (64%)	70 (52%)	93 (44%)	352 (55%)	78%

Group 1 120th Station Hospital Army Hepatitis Center Bayreuth  
 Group 2 387th Station Hospital Army Orthopedic Center Stuttgart.  
 Group 3 Patients 79th Station Hospital Berlin  
 Group 4 Detachment personnel 29th Station Hospital Berlin  
 Group 5 Indigenous personnel employed in Army messes

†Large races only 20 large and small races together 3 small races only 31 Cysts only 44 cysts and trophozoites 1 trophozoites only 9

†Parasitized individuals harbored from one to five species

Combined in Table I with 1492 indigenous personnel surveyed between July 1946 and November 1947. By indigenous personnel here is meant Germans employed in Army in installations

†Twenty persons included in a preliminary report<sup>2</sup> are here omitted because they either gave a foreign home address or gave no home address

†Including twenty five United States civilians and dependents

Three techniques were employed on each stool (1) iron hematoxylin stained smears, (2) zinc sulfate centrifugal concentration, and (3) thick fecal smears, fixed and dehydrated in 95 per cent and 100 per cent alcohol and cleared in xylol. One zinc sulfate concentration, two hematoxylin smears, and two transparent thick smears were examined for each stool. Each person was given a saline purge and requested to submit a warm, soft, unformed stool sample, with a completed questionnaire. One stool sample from each person was examined. The same procedure was followed in all surveys previously conducted on indigenous food handlers in Army messes.

### RESULTS

A tabular presentation of the survey is shown in Table I. The possible effect of disproportionate numbers of persons with previous residence in "southeastern states" of the United States and with tropical service in the various groups and the groups in which trophic forms of protozoa might exist are indicated in Table II. While only 50 per cent of the stools obtained at the Hepatitis Center were actually unformed, a large percentage of the formed stools were soft and were promptly delivered to the laboratory. The material obtained at both Bayreuth and Stuttgart was quite comparable with that obtained from indigenous personnel for several previous surveys.

TABLE II INCIDENCE OF PARASITISM IN RELATION TO THE PERCENTAGE OF PERSONS FROM SOUTHEASTERN STATES AND WITH TROPICAL SERVICE EXAMINED, AND THE NATURE OF THE STOOL SAMPLE EXAMINED

	INCIDENCE (%)	INFESTATIONS 1,000	PER CENT FROM SE STATES	PER CENT WITH TROP ICAL SERVICE	PER CENT UNFORMED STOOLS
Stuttgart	64	1,252	45	23	80
Bayreuth	60	1,028	41	16	50
Berlin, patients	52	798	37	18	35
Berlin, detachment personnel	44	743	34	13	24
Mean	55	935	38.8	16.8	45

Table III shows the incidence of parasitism among persons from southeastern states as compared with that of persons from all other states combined. Thirty-two (13 per cent) of 250 persons who gave a permanent address in a southeastern state harbored hookworm. Fifteen persons (6 per cent of 250) harbored no other parasites.

One hundred and eight (16.8 per cent of 644) persons had served in the tropics. Sixty per cent harbored intestinal parasites. Sixteen (21.6 per cent) of 74 persons who harbored *Endamoeba histolytica* had been in the tropics.

TABLE III INCIDENCE FOR PERSONS FROM SOUTHEASTERN STATES AS COMPARED WITH PERSONS FROM OTHER STATES

	SOUTHEASTERN STATES (%)	OTHER STATES (%)
Stuttgart	66	62
Bayreuth	69	54
Berlin, patients plus detachment personnel	63	39
Mean incidence	65	47

\*Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, Virginia, West Virginia. District of Columbia, Arkansas, Oklahoma, and Texas are included because of the presence of hookworm in certain areas.

†One person from California (with tropical service) and one person from the state of Washington (with no tropical service) also harbored hookworm.

The incidence for persons (all groups combined\*) who had been in Germany one year or less was 50 per cent from not less than one year to two years, 55 per cent, and for more than two years, 48 per cent. In a group of 224 persons who were from states other than southeastern and who had not been in the tropics, incidences were, for these respective periods, 51 per cent, 47 per cent, and 55 per cent.

Fifteen (43 per cent) of 35 colored soldiers harbored parasites.

Seven women patients, 6 of whom harbored parasites, are included in the Berlin survey.

Of 633 persons who stated their age, 36.2 per cent were from 17 to 20 years of age, 30.6 per cent, from 21 to 25, 16.8 per cent from 26 to 30, and 16.4 per cent were over 30 years of age. In the hepatitis group alone the percentages in these respective age groups were 40 per cent, 25.7 per cent, 20.6 per cent, and 14.3 per cent, and the percentages harboring intestinal parasites in these age groups were 62 per cent, 69 per cent, 57 per cent, and 53 per cent. For all surveys combined the percentages harboring parasites in the four age groups were 53 per cent, 62 per cent, 52 per cent, and 43 per cent.

Sixty-five persons (10.1 per cent) of 644 stated they had had, at the time of the stool examination or within the recent past, one or more episodes of diarrhea. Thirty-nine of these 65 were in the group with hepatitis. Thirty-four (52 per cent) of the 65 harbored intestinal parasites. The percentage of persons with a history of diarrhea was for each group as follows: hepatitis patients, 18.4 per cent, orthopedic patients, 3.6 per cent, Berlin patients (all services), 5.8 per cent, Berlin detachment, 6.4 per cent. Of those harboring *E. histolytica*, 31.4 per cent gave a history of diarrhea. For other organisms the percentages were: *Giardia lamblia*, 17.6 per cent, *Chilomastix mesnii*, 25 per cent, *Dientamoeba fragilis*, 4.4 per cent, hookworm, 8.8 per cent.

The patients and detachment personnel at Berlin may be considered as representative residents of that particular area. The patients at the Hepatitis and Orthopedic Centers are drawn from all parts of the American Zone of Occupation.

# DISCUSSION

As an oblique approach to the problem of the transmission of "infectious hepatitis" in Germany—one aspect of which may be the contamination of food and water with fecal material—surveys for intestinal parasites have been made on 644 healthy and hospitalized American personnel. The incidence of parasitism in the group with hepatitis was 60 per cent and in a group of orthopedic patients in another hospital the incidence was 6.4 per cent. A third group of hospitalized persons (all services) had an incidence of 52 per cent, and the incidence in a group of hospital detachment personnel was 4.4 per cent. An average of 55 per cent harbored from one to five species of parasites.

The variations in incidence found are probably attributable more to such variable factors as former residence in the southeastern part of the United

\*Persons with hookworm only. In this instance were regarded as showing negative results since the acquisition of hookworm in Germany is highly unlikely.

States, tropical service, and, perhaps mainly, the character of the stool sample examined than to any considerable difference in the amount of fecal contamination of food and water to which any one group had been exposed

There may be a real difference in the incidences for persons from south eastern states and from other parts of the country, partly attributable to hookworm infestations. However, in the group that contained the highest percentage of persons from southeastern states, and which submitted the most suitable material for stool examinations, no difference is to be noted

The average incidence for 1,705 indigenous personnel was 78 per cent, with parasitized individuals harboring from one to seven species. If the incidence of parasitism among Americans has been affected by environmental conditions in Germany or by contact with indigenous personnel, it is not apparent in the present study. No appreciable increase or decrease in incidence with continued residence in Germany was found

#### SUMMARY

1 A survey of intestinal parasitism among American Occupation Forces in Germany and indigenous German personnel is presented and compared with the incidence of intestinal parasitism in patients suffering from acute and chronic hepatitis. No significant difference between patients with hepatitis and other groups was noted

2 The average incidence of intestinal parasitism among hepatitis patients was 60 per cent, while the average in other hospitalized patients was 55.3 per cent. The average in apparently healthy American soldiers was 44.0 per cent, while in indigenous personnel it was 78 per cent. An analysis of the individual parasites in each group is presented

3 A discussion of the significance of these findings in relation to other factors is presented

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# PRIMARY HISTOPLASMOSIS WITH RECOVERY OF HISTOPLASMA CAPSULATUM FROM THE BLOOD AND BRONCHIAL SECRETIONS

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**D**URING the course of studies on the significance of skin sensitivity to fungus antigens of children with acute respiratory infections and pneumonia,<sup>1</sup> we had the opportunity to observe the clinical course and carry out bacteriologic and serologic studies in a case of primary pulmonary histoplasmosis in a 12 year-old colored boy. We believe that this case is of particular significance because it adds further support to increasing reports concerning the benign nature of histoplasmosis and completes the missing picture comprising the initial infection and progress through the phase of healing. The infection was followed from the onset to complete recovery with beginning calcification. As far as we know, this is the first case of proved active primary pulmonary histoplasmosis to be reported in a child who was born and reared in the Chicago area.

## CASE REPORT

J G, a 12 year old colored boy who has been observed in the outpatient cardiac clinic for the past four years following an attack of rheumatic fever with carditis, was admitted to Sarah Morris Hospital on Sept 4, 1948, with a tentative diagnosis of recurrent acute rheumatic fever. This patient was born and reared in Chicago and never left Illinois. He developed rheumatic fever and carditis at the age of 9 years. He made a good recovery but was left with mitral insufficiency and some cardiac enlargement. During the past three years he attended a summer camp for cardiac patients and has been in good health. During the years preceding the attack of rheumatic fever he had measles and mumps. The family history was noncontributory except that a sister, 12 years old, died in April, 1944, of rheumatic heart disease. Also a brother, 18 years old, died in March, 1948, with a clinical diagnosis of pulmonary tuberculosis, but no autopsy was done.

Five days before admission, the patient had returned from a summer camp for cardiac patients located near Chicago. On the day of his return he complained of a head cold and headache. He had a slight nonproductive cough and a fever. The day before admission to the hospital he complained of pain in his left elbow, unaccompanied by swelling.

Physical examination revealed a well developed and well nourished colored boy of 12 years who did not appear acutely ill. The temperature was 102.6° F, respiratory rate, 28 per minute, and the pulse rate, 82. The positive findings were moderate nasal congestion, slightly enlarged anterior cervical lymph nodes, slight cardiac enlargement to the left, a loud blowing systolic murmur at the apex which was transmitted to the axilla, and an early diastolic murmur at the apex and base. The lungs were clear, the liver and spleen were not enlarged, and the extremities appeared normal. The laboratory findings are summarized in Table I.

An x ray of the chest on September 7 revealed clear lung fields and slight cardiac enlargement (Fig 1). Skin tests done on September 7 with histoplasmin\* and tuberculin gave positive reactions with 7 mm and 13 mm indurations respectively, while skin tests

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\*Fungus antigens were used in dilutions of 1:10000. PPD was used in dilution of 0.0001 milligram.



TABLE I SUMMARY OF LABORATORY DATA

DATE	9/4/48	9/7/48	9/14/48	11/17/48	12/7/48
Red cell count	3.6 mil	—	4.0 mil	3.9 mil	3.9 mil
Hemoglobin	11.2 Gm	—	11.2 Gm	11.9 Gm	12.6 Gm
White cell count	5,900	—	5,200	6,500	6,000
Differential count (%)					
Neutrophils	14	—	50	52	69
Lymphocytes	7.5	—	38	30	23
Eosinophils	3	—	1	2	0
Basophils	1	—	0	1	0
Monocytes	7	—	11	15	8
Urinalysis	Negative	—	—	Negative	—
Sedimentation rate (mm per hr)	28	15	—	24	10
Wassermann and Kahn	Negative	—	—	—	Negative
Antistreptolysin titer	—	166	—	100	—
Weltmann reaction	—	5	—	5	—
Blood cultures	Negative	—	—	II capsula tum iso lated	—

with blastomycin, coccidioidin, and torulin were negative. On symptomatic treatment the patient's temperature became normal after forty-eight hours and he made an uneventful recovery. There were no significant changes in the cardiac findings. The patient was discharged from the hospital on Sept. 15, 1948, eleven days after admission.

**Second Admission.**—The patient was readmitted to the hospital on Nov. 17, 1948, with a history of malaise and fever for one day. The temperature was 104° F, pulse rate, 104, and respiratory rate per minute, 22. The blood pressure was 130 mm of mercury systolic and 62 mm diastolic. Pertinent findings were injected pharynx, moderate injection of both eardrums, and cardiac findings indicative of mitral stenosis and regurgitation and aortic regurgitation. The pertinent laboratory data are shown in Table I.

The patient was given penicillin and salicylates. His temperature dropped from 104° F on the day of admission to a maximum of 101° F on the following day. Subsequently, a low grade fever up to 100.2° F persisted for an additional eight days, the total duration of fever being ten days. During this period the patient did not appear ill despite the fact that a large pulmonary infiltration was observed in the left lung on November 18 (Fig. 2). Occasionally he had a slight nonproductive cough. On November 24 physical findings indicative of pulmonary disease were observed for the first time, consisting of slight dullness and increased breath sounds at the base of the left lung. No rales were heard. Although the patient was asymptomatic ten days after admission to the hospital, x-rays of the chest showed exceedingly slow clearing, and at the time of discharge on December 17, there was only about 50 per cent clearing of the pneumonic process (Figs. 3 and 4). An x-ray of the chest taken on Jan. 4, 1949, revealed further clearing and a soft nodular density in the fifth left interspace. Furthermore, a small area of greater density was present in the sixth left interspace which appeared to be beginning calcification (Fig. 5). On March 21, the infiltration had completely disappeared and minute areas of calcification were present in both the fifth and sixth left interspaces (Fig. 6). The last film taken on May 9 showed no significant changes as compared with that of March 21. Skin tests performed on Dec. 1, 1948, with histoplasmin and tuberculin showed 20 mm and 10 mm indurations respectively, while tests with blastomycin, coccidioidin, and torulin remained negative.

#### BACTERIOLOGIC STUDIES

On admission the throat culture was positive for *Staphylococcus aureus*, and no fungi were isolated. Blood cultures were negative. A blood culture obtained on November 17 during the second admission was positive for *Histo plasma capsulatum* in the yeast phase after incubation in beef infusion broth.



Fig 1—Roentgenogram of the chest taken Sept 7 1948 at the time of first admission. The hilar lymph nodes appear larger than normal. The lung fields are clear.

Fig 2—Roentgenogram of the chest taken Nov 18 1948 at the time of second admission. There is a large parenchymal infiltration in the left mid-lung field. There is little change in the appearance of the hilar lymph nodes.



Fig 3—Dec 9 1948. Since the previous examination of Nov 18 1948 there has been about 50 per cent clearing of the infiltrative process in the left lung.

Fig 4—Dec 15 1948. Since the examination of Dec 9 1948 there has been only light clearing of the infiltration in the left lung.

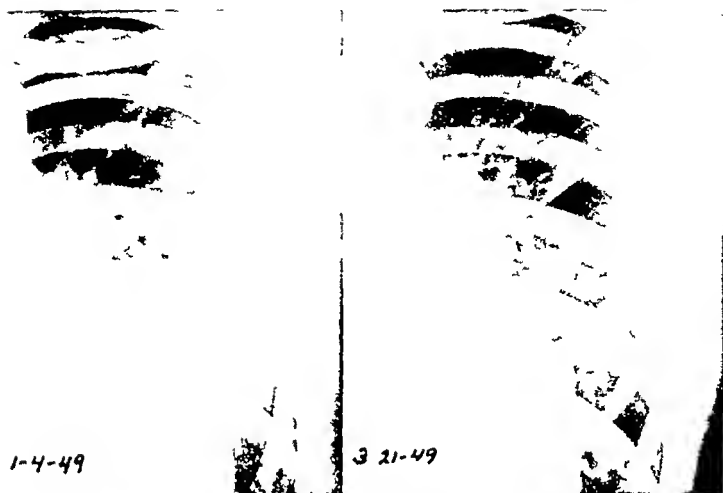


Fig —Jan 4 1949. There is still considerable infiltration in the left mid lung field. There is an area of focalization in the region of the fifth left interspace. In the sixth left interspace there is a small circumscribed area of greater density that appears to be beginning calcification.

Fig 3—March 21 1949. There is complete clearing of the infiltration in the left lung. Minute areas of calcification are present in both the fifth and sixth interspaces. There is an increased amount of calcification in both hilar areas.



Fig —tuberculate chlamydospores of *Histoplasma capsulatum* recovered from the blood on Nov 17 1948

for seven weeks at 37° C (Fig 7) No bacteria were isolated A throat culture at this time showed anhemolytic streptococci and *Alcaligenes faecalis* Repeated gastric washings were negative for acid-fast bacilli or pathogenic fungi in direct smear and culture Bronchoscopic specimens obtained on December 10 were negative for acid-fast bacilli in smear and culture However, one of six mice inoculated with bronchial material and sacrificed six weeks later showed lesions in the liver and spleen from which *H capsulatum* was recovered

#### SEROLOGIC STUDIES (TABLE II)

Complement fixation tests with undiluted serum carried out on December 1 showed a 4+ reaction with the histoplasmin antigen (lot H-15, 1:100 dilution), on December 20 the reaction was 3+ while on April 2, 1949, the reaction decreased to 2+ The technique described by Tenenbeig and Howell<sup>2</sup> and Fuleow and co-workers<sup>3</sup> was employed in the complement fixation tests Complement fixation tests carried out simultaneously with blastomycin antigen gave negative results Virus and rickettsia complement fixation tests for psittacosis, pneumonitis group, Q fever, and influenza A and B on some specimens of this sera were repeatedly negative Serum drawn on December 22 was positive in 1:20 dilution (4+) with lymphocytic choriomeningitis antigen but was negative with serum drawn on April 2 Repeated tests for heterophile and cold agglutinins were negative

TABLE II SUMMARY OF VARIOUS COMPLEMENT FIXATION AND OTHER SEROLOGIC TESTS

DATE	11/26/48	12/1/48	12/20/48	12/22/48	4/2/49
Histoplasmin	—	4+*	3+*	—	2+*
Blastomycin	—	0	0	—	0
Psittacosis pneumonitis	—	—	—	0	0
Lymphocytic choriomeningitis	—	—	—	4+†	0
Influenza A and B	—	—	—	0	0
Q fever	—	—	—	0	0
Cold agglutinins	0	0	—	0	0
Heterophile	0	0	—	0	0

\*Undiluted serum

†Serum diluted 1:20

#### DISCUSSION

Although a great deal has been written concerning benign histoplasmosis, pulmonary calcification, and recovery from systemic histoplasmosis, relatively little has appeared describing in detail the previously hypothesized primary phase, which in all probability represents the link between the initial infection and pulmonary calcification Mention is made of noncalcified pulmonary infiltrates by Dickie and Clark,<sup>4</sup> but these authors failed to describe the initial infection and the progress of the lesions

Sontag and Allen<sup>5</sup> recently correlated the presence of calcific and precalcific pulmonary lesions with histoplasmin sensitivity in a study of 170 "normal" children These authors, however, mention no attempt to isolate an etiologic agent Serial x-rays were made over a period of many months and it was possible to study progressive changes in the lungs from the onset of the initial lesion through the period of calcification Their studies showed "a

tendency for calcification associated with positive histoplasmin reactions to be (1) multiple in the parenchyma often with calcifications present in upper lung fields and apices, (2) early in onset frequently with the initial lesion appearing before one year of age, the majority before 4 years of age, (3) preceded occasionally by a pneumonic infiltrate early in the infection and (4) progressive with the development of new lesions and calcifications over a period of years "

In discussing the possibilities of secondary infection with histoplasmosis, Peterson<sup>6</sup> states that in some instances it is almost impossible clinically to differentiate the progressive pulmonary form from pneumonia. Fureolow states<sup>7</sup> that areas of pneumonitis are seen in children in relationship to the development of histoplasmin sensitivity. Although these areas of pneumonitis were often asymptomatic, occasionally they were associated with definite clinical symptoms especially where there had been marked regional adenopathy and associated pressure reactions. Some of the lesions have the appearance one would expect around a focal granulomatous lesion at the time sensitivity develops. He further states that these lesions are uncommon and that it is often difficult to establish the diagnosis.

In a study of 16 000 school children in Kansas City, Fureolow, Mantz, and Lewis<sup>8</sup> described the roentgenologic characteristics of persistent pulmonary infiltrations in 72 asymptomatic patients who were sensitive to histoplasmin but not tuberculin. They found that only a few were limited to the lymph nodes, a few were of the disseminated type, while approximately two thirds were nodular, sharply circumscribed foci. The remaining one fourth were diffuse, patchy infiltrations with poorly defined borders, which sometimes developed into nodular lesions. Similar lesions were seen in a few patients whose symptoms appeared to arise from this infection. In some of the lesions a central core of calcification was noted initially or in subsequent films. The lesions tended to calcify slowly, and many infiltrations persisted without complete calcification during the two years of observation.

These authors also stated that up to the present time they had no cases in which the entire progress of events could be observed, i. e. negative chest x-rays, negative tuberculin and histoplasmin sensitivity through the appearance of the parenchymal infiltrate with concurrent change in cutaneous sensitivity, to the final irreversible calcified focus in the lung or node associated with a positive histoplasmin test (and negative tuberculin test). In all probability a longer period of observation would be necessary in order to observe the final period of calcification. However, our patient began to show calcification approximately 4 months after the appearance of the pulmonary infiltrate. They further state that they have seen patients with initially negative x-rays and histoplasmin reactions who later developed pulmonary infiltrates or hilar adenopathy or both together with the development of a positive cutaneous reaction to histoplasmin.

We feel that our case represents the precursor phase of pulmonary calcification and, therefore, completes the hypothetical stage proposed by others. We

were able to demonstrate the progress of the primary phase of histoplasmosis presumably from the onset of the first symptoms and roentgenologic evidence of pulmonary infiltration to final clearing of the lesion and beginning calcification, correlating the clinical progress of the disease with bacteriologic and serologic findings. Radiologic evidence of consolidation was present on Nov. 18, 1948 (Fig. 2), three days after the onset of symptoms and eighty days after the onset of the primary illness (Aug. 31, 1948, when the histoplasmin skin test was positive). It is unfortunate that we failed to do a complement fixation test at this time so that it is difficult to determine exactly when the primary infection took place. Nevertheless, on the basis of the 2+ histoplasmin reaction on November 18 and the subsequent increase in sensitivity associated with the strongly positive complement fixation test, in all probability the primary infection occurred shortly prior to August 31. Thus, one can postulate that the pulmonary infiltration occurred at least eighty-five to ninety days or more from the time of the initial infection.

In our case there may be some question as to the significance of the positive tuberculin test. In all probability there is no correlation between the positive tuberculin and histoplasmin tests. Had we not obtained a positive blood culture and isolated *H. capsulatum* from a mouse inoculated with bronchial secretions, the diagnosis of histoplasmosis and the significance of the positive histoplasmin test would be doubtful.

Fungus skin tests were performed on other members of the family, all of whom are free of pulmonary disease. The mother was histoplasmin and tuberculin positive, and the complement fixation test was negative. The three siblings, 23, 15, and 10 years of age respectively, were positive to tuberculin and negative to all of the fungus antigens. This suggests that in all probability all had a common exposure to tuberculosis.

The complement fixation test apparently is important in distinguishing active and latent or past infections with *H. capsulatum*. Those with recent changes from negative to positive skin sensitivity seem more likely to show positive complement fixation, while those with latent or healed infections are generally negative. The finding of a positive complement fixation test for lymphocytic choriomeningitis (see Table II) was probably due to past infection. No virus was isolated from blood or bronchial secretions obtained during the acute stage and inoculated into mice and guinea pigs. Three other members of the patient's family who were tested at approximately the same time also had positive complement fixation tests for the choriomeningitis virus. The family gave a history of living in a house about six months previously that was overrun with mice. Moreover the complement fixation test became negative when repeated with serum drawn from our patient 101 days after the positive specimen.

#### SUMMARY

A case of primary histoplasmosis in a 12-year old colored boy, with clinical findings of atypical pneumonia, is described. The course of the infection was followed from the onset through the stage of pulmonary infiltration to healing.

as manifested by beginning pulmonary calcification. *H. capsulatum* was isolated from the blood and bronchial secretions obtained during the acute stage. The patient had a positive complement fixation test for histoplasmosis at the height of the pulmonary infiltration which persisted to the time of beginning calcification four months later. He also had a positive histoplasmin skin test. Benign histoplasmosis is discussed with particular reference to precalcific pulmonary infiltrations.

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## LABORATORY METHODS

### A NEW MOUNTING FOR THE ELECTROKYMOGRAPH

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IN 1945 Henny and Boone<sup>1</sup> successfully employed an electron multiplier phototube (RCA, 931-A) as a pickup device for the registration of heart motion, using the fluoroscope to delineate the cardiac silhouette. Motion of the heart borders was translated into a varying electric current which was, in turn, recorded as a deflection tracing on an electrocardiograph of either the string or D'Aisonval type. The apparatus was termed an "electrokymograph." Subsequently Henny, Boone, and Chamberlain<sup>2</sup> developed an improved mounting for the phototube which facilitated its use with the ordinary fluoroscope. In 1948 Luisada and co-workers<sup>3</sup> modified this apparatus and called their device a "fluorocardiograph."

In both instruments positioning of the pickup device slit with respect to a given part of the cardiac silhouette is of paramount importance. This was accomplished by both groups through the use of a knurled knob which rotated the phototube pickup by means of a pulley and belt. The knob projected from the operator's side of the screen. In the model used by Henny, Boone, and Chamberlain,<sup>2</sup> the phototube was permanently mounted on a bracket which could be rotated out of the way for routine fluoroscopy. The Sanborn model, employed by us, is constructed according to the modifications developed by Luisada and co-workers and consists of a pantograph arm which clamps to the fluoroscopic screen. Our experience has proved this arrangement to be unwieldy. The phototube tends to rotate after it is positioned and the joints of the pantograph arm give way from the weight of the pickup device. In addition, it was found that the cables of the phototube head frequently dislodged the microphone from its position on the chest when heart sounds were being recorded simultaneously with the electrokymograph.

With these difficulties in mind, we mounted the Sanborn pickup device in the center of an aluminum frame which slides into the existing channels of the 1704 A Picker spot-film device belonging to our x-ray unit. This has the advantage of enabling the entire frame and phototube to be readily slid out of the fluoroscopic field when not in use, when the operator has located the heart border desired, a simple movement of the lead frame holder moves the pickup rapidly into position, and thereafter but minor adjustments of the pickup slit are necessary.

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Fig 1—A photograph of the electrokymograph pickup device in the new radiolucent aluminum and plastic mounting Described in text.

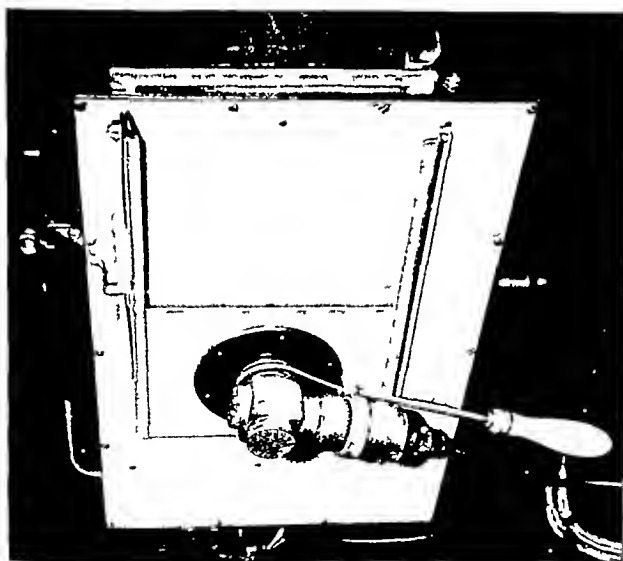


Fig 2—The new mounting fitted into the spot film device

Positioning of the slit through 180 degrees is accomplished by a long arm (parallel with the slit) similar to that used on a camera mounting. Rotating this arm through a 180 degree arc allows fine orientation of the slit with respect to the cardiac silhouette. Further motion of the phototube is prevented by a screw at the phototube mounting which is operated by turning the handle of the arm. The entire fluoroscopic screen and tube carriage can be locked in any position by the brake on the x-ray unit. Since this is a spot

radiographic device, it is possible to obtain 5 in by 7 in spot films of the phototube pickup head in position on the cardiac silhouette for purposes of permanent record. Figs 1 and 2 are photographs of this new modification.

#### SUMMARY

1 A new mounting for the electrokymograph by use of a spot-radiographic device is described.

2 The advantages of this modification are presented.

We are indebted to Mr N G Weidman of Picker X ray Corporation for valuable suggestions.

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## METHODS FOR THE DETERMINATIONS OF RADIOACTIVE PHOSPHORUS ( $P^{32}$ ) IN BODY FLUIDS

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WITH THE TECHNICAL ASSISTANCE OF MARY I. KENDRICK, B. S.

THE use of radioactive isotopes in tracer amounts in man imposes the problem of the determination of very small quantities of isotopes in the body tissues and fluids. This is particularly true when tracers such as  $P^{32}$  are used since the over all distribution, excretion, and exchange of phosphate are large and rapid. To meet this situation selection and modification of existing methods for the isolation and estimation of the various phosphate fractions, both radioactive and nonradioactive are required.

The purpose of the present communication is to present the techniques developed for the investigations of phosphorus metabolism in man using  $P^{32}$  as a tracer in doses of 100 to 200 microcuries<sup>1</sup> intravenously.

### APPARATUS

The  $P^{32}$  beta radiation<sup>2</sup> was counted with end window (bell type) Geiger Muller tubes and a Tracerlab Autoscaler. Both commercially manufactured Geiger Muller tubes and those assembled by the Massachusetts Institute of Technology were employed. The mean window thickness varied from 2 to 5 mg per square centimeter. The tubes were shielded with 3 to 6 in. of lead. The background varied from 0.23 to 0.45 counts per second for different tubes, but was essentially constant for any one tube during the period of study. For any given set of experiments one tube was used. The critiques of measurement were those reviewed by Kamen.<sup>2</sup>

Calibration of the Autoscaler was made daily by the use of a standard 60 cycle alternating current and the complete assembly was checked by the use of a uranium standard. A manual sample changer was constructed so that each sample was at a fixed and constant distance from the Geiger Muller tube window. To ensure maximum recording this distance was the minimum required to prevent contamination of the tube by the sample.

### MATERIALS

Only dried samples were used and to approximate similar distribution of materials in all samples the amount of liquid dried was kept constant throughout these investigations. Since 2 ml. was found to be the minimum volume with which accurate counts could be obtained for the least active samples the more active samples were diluted so that 2 ml. contained an appropriate activity.

Bottle caps<sup>3</sup> prior to the insertion of the cork inlay were used as expendable containers for drying samples. Occasionally such bottle caps were delivered prevarnished. It was found necessary to remove the varnish with a concentrated solution of sodium hydroxide. All bottle caps were washed first with acetone to remove machine oil and grease and subsequently with distilled water and then dried.

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<sup>1</sup> $P^{32}$  was supplied by Monsanto Chemical Company Clinton Laboratories Oak Ridge Tenn.

<sup>2</sup>From the Armstrong Company Lancaster Pa.

Standards were prepared by drying 2 ml samples of appropriate dilutions prepared from the solutions of  $P^{32}$  which were injected into subjects or added to in vitro reaction mixtures. By means of these, corrections for decay of the isotope could be made.

The method used for drying samples varied depending on the physical characteristics and composition of the substance under investigation. The use of the infrared lamp as a source of heat provided the most convenient and rapid way of drying samples. Unfortunately, it could not be used when the sample contained more than a very small amount of organic matter. When plasma, whole blood, or trichloroacetic acid filtrates were dried at a high temperature, the residues were uneven and often elevated, and considerable error in counting resulted. It was found that when samples containing organic matter were dried overnight at 38 to 40° C more evenly deposited dried samples, closely adhering to the sample containers, were obtained.

*Great care must be employed to be certain that the samples are thoroughly dry, since the presence of even small amounts of moisture has been found to lower the counting rate significantly.* After most of the moisture had been removed at 38 to 40° C, heating under an infrared lamp or in a hot oven at about 100° C for one to two hours could be carried out to ensure complete dryness, without causing irregularities in the deposited material.

The thickness, consistency, and density of the samples are important factors in the accurate estimation of radioactivity because of their well known effect on the absorption of the emitted beta rays. Ideally, all phosphorus containing fractions should be separated by suitable chemical methods and then converted by chemical procedures to inorganic phosphorus so that direct comparisons of the radioactivity could be made on the same final substance. However, with the use of tracer amounts of  $P^{32}$  in man, separation and conversion to inorganic phosphate by organic combustion would introduce dilutions so great as to reduce the radioactivity in some of the fractions of phosphorus to the point where unfeasibly long periods of time would be required for accurate counting. Therefore, no attempt was made to convert all fractions to the inorganic form. As a result, measurements were made on dried samples of different physical characteristics. This was dictated by the necessity of using the maximum amounts of material in the samples consistent with ease in preparation and counting. Since the samples consisted of rather large amounts of material in nonstandard geometry and unknown uniformity, the corrections for selfabsorption and other physical properties were made empirically. These measurements were also used as a check upon the reproducibility of the data. These corrections will be discussed below in detail for each fraction.

## METHODS

Oxalated blood samples, cooled in ice, were centrifuged as soon as possible after the blood was drawn, and the plasma removed as completely as possible. The red blood cells were washed once with ice cold isotonic sodium chloride solution, centrifuged again, and the supernatant washings removed as completely as possible and discarded. The washed red blood cells were frozen to prevent hydrolysis of phosphorus compounds and to produce hemolysis. Plasma and thawed hemolyzed red blood corpuscles were analyzed chemically for total phosphorus, total acid soluble phosphorus, and inorganic phosphorus, and the radioactivity of these phosphate fractions was determined. Corresponding values for the total organic, acid soluble organic, and nonacid soluble organic fractions were calculated.

Quantitative volumetric measurement of samples of hemolyzed red blood cells was somewhat difficult because of their high viscosity. Probably the most accurate way of measuring them is by weight. This technique is indeed necessary if undiluted red blood cells must be used. However, as a technique for handling many samples of red blood cells, weighing is extremely time consuming. Accordingly, the thawed red blood cells were measured with Ostwald Folin pipettes calibrated to deliver by blowing out the last drop and transferred to volumetric flasks of desired capacity. The red cells adhering to the sides of the pipette were removed by repeated washings with distilled water, the washings were added quantitatively to the volumetric flask containing the pipetted cells, and the mixture was made up to the desired volume with distilled water. Samples from dilutions of five times the original volume of red cells can be pipetted accurately without difficulty and with good duplication of results.

1 *Total Phosphorus in Plasma and Red Blood Cells*—The total phosphorus concentration of plasma and red blood cells was determined in samples of suitable size by the colorimetric molybdate method after digestion with sulfuric and nitric acids, as described by Fiske and Subbarow.<sup>3</sup>

The total  $P^{32}$  content of plasma was determined by counting the beta rays from 2 ml samples of plasma dried overnight at 38° C.

For the determination of the  $P^{32}$  content of erythrocytes a 1:5 dilution of hemolyzed red blood cells was prepared and 2 ml samples of this dilution were dried overnight at 38° C. The beta particles emitted were then counted.

2 *Total Acid Soluble Phosphorus of Plasma and Red Blood Cells*—To precipitate the plasma proteins, 1 vol of plasma was mixed with 4 vol of 10 per cent trichloroacetic acid. The mixture was shaken well and filtered through Whatman No. 42 filter paper.

In precipitating the protein from red blood cells a 1:10 dilution was made instead of the 1:5 dilution used for plasma in order to permit adequate washing of the pipette used to measure the cells. Five milliliter samples of hemolyzed red blood cells were pipetted into 50 ml volumetric flasks. The pipette was thoroughly washed with water and the washings were transferred quantitatively to the same volumetric flask. Ten milliliters of 20 per cent trichloroacetic acid were added with shaking. The contents of the volumetric flasks were made up to 50 ml with distilled water mixed thoroughly by inversion and filtered through Whatman No. 42 filter paper. This preparation contained the same weight of trichloroacetic acid per milliliter of red blood cells as was used per milliliter of plasma but the final dilution was twice as great in respect to both red cells and trichloroacetic acid. Since it was difficult to adjust the volume accurately because of foam and floating particles of precipitated protein, two preparations of red blood cells were routinely made and a sample was taken from each filtrate for all determinations made on the protein free filtrates.

The total acid soluble phosphorus in the trichloroacetic acid filtrates prepared from plasma and red blood cells was determined by the colorimetric molybdate method of Fiske and Subbarow.<sup>3</sup>

To determine the  $P^{32}$  present in the acid soluble phosphate fractions 2 ml samples of the trichloroacetic acid filtrates obtained from both plasma and red blood cells were transferred to bottle caps and dried overnight at 38° C. The emitted beta particles were then counted.

3 *Inorganic Phosphorus in Plasma and Red Blood Cells*—The inorganic phosphorus of the plasma and red blood cells was determined in the protein free trichloroacetic acid filtrates by the colorimetric molybdate method of Fiske and Subbarow.<sup>3</sup>

For the measurement of  $P^{32}$  in the inorganic phosphate fraction of plasma and red blood cells, the inorganic phosphate was separated from the other forms of acid soluble phosphate by the calcium precipitation method of Fiske<sup>4</sup> with certain modifications and precautions which were found necessary for the final counting techniques.

Twenty milliliter samples of the trichloroacetic acid filtrate prepared as given in the section on acid soluble phosphorus were pipetted into 25 ml volumetric flasks. One or 2 drops of phenolphthalein were added and the filtrate was carefully neutralized with, first, 70 per cent, then normal, and finally,  $\frac{1}{10}$  normal sodium hydroxide until a small drop of the  $\frac{1}{10}$  normal alkali sufficed to produce a faint pink color. If the end point was passed with one of the stronger alkali solutions trichloroacetic acid was added until the solution was colorless and the end point established again with  $\frac{1}{10}$  normal alkali. It was found to be of the greatest importance to avoid an excess of alkali because of subsequent failure to obtain complete solution of calcium phosphate.

The neutralized filtrate was made up to exactly 25 ml in the volumetric flasks and thoroughly mixed. Two 10 ml samples were measured into conical graduated 15 ml centrifuge tubes. One tenth milliliter of 0.4 per cent sodium phosphate (approximately 0.1 mg of phosphorus) was added to the solution in the centrifuge tube to act as a carrier for the  $P^{32}$  during subsequent manipulations.

Five milliliters of a 10 per cent solution of calcium chloride, saturated with calcium hydroxide were then added to the contents of the centrifuge tubes and the mixture was allowed to stand for ten minutes to ensure complete precipitation of the phosphate as calcium phosphate.

The tubes and contents were then centrifuged for two minutes at 3,000 revolutions per minute. *This speed of centrifuging was found to be absolutely necessary for the quantitative separation of the precipitate from the supernatant liquid.*

The supernatant fluid was carefully decanted from the precipitate and the tubes were drained by standing them inverted on filter paper for a few minutes.

The precipitate was washed once by resuspending in a mixture of 4 ml of distilled water and 1 ml of the  $\text{CaCl}_2 \text{Ca}(\text{OH})_2$  reagent and recentrifuged at 3,000 rpm for two minutes. The supernatant liquid was decanted and the tubes again were drained by inversion.

Two drops of normal sulfuric acid were added to the calcium precipitate. The gelatinous precipitate of calcium phosphite was replaced by a white amorphous precipitate of calcium sulfate. Repeated tests have shown that all of the phosphate ion at this stage is in solution.

The contents of the 15 ml centrifuge tube were made up to 5 ml with distilled water, stoppered, and thoroughly shaken. The stoppers were removed and the tubes centrifuged for about five minutes at approximately 2,000 revolutions per minute.

Two milliliter samples were pipetted from the supernatant fluid into bottle caps and dried. Because of the low salt content and the absence of organic matter in the sample, drying was done rapidly under an infrared lamp. The emitted particles from the dried samples were then counted.

4 *Urine*—Urine samples were collected for various intervals of time depending on the periods of most interest in each individual in vivo experiment. The total volumes were measured, and 2 ml samples of urine were dried and counted with the Geiger Muller counter and Autoscaler. Following the injection of 100 microcuries of  $\text{P}^{32}$ , the urine of normal subjects was found to contain enough radioactive material so that 2 ml samples, diluted or undiluted, gave counts within an accurate range for five or six days following the administration of the isotope. In the first hours the counts were so high that dilution was carried out in order to avoid counting errors while maintaining the 2 ml volume of sample.

TABLE I STATISTICAL ANALYSIS OF COUNTING DRIED AQUEOUS SOLUTIONS OF  $\text{P}^{32}$

MEAN* (CPS)	STANDARD DEVIATION (CPS)	MEAN* (CPS)	STANDARD DEVIATION (CPS)
150	± 07	288	± 46
161	11	289	52
274	05	291	43
440	19	295	70
445	19	301	32
465	14	305	64
469	23	326	18
510	18	337	30
804	25	349	96
95	26	393	86
118	14	423	67
122	27	433	117
124	40	441	118
125	36	441	95
140	00	454	86
149	59	523	59
155	82	673	224
165	24	1017	245
189	72	1047	186
189	86	1206	439
198	80	2108	404
210	51	2200	550
211	63		
214	95		
242	64		
259	94		
264	77		
280	51		

CPS Counts per second

Standard deviation of the mean for the entire series is  $\pm 3$  per cent

\*Each figure in the column Mean (CPS) is the mean of six readings, three on each of two duplicate samples

*Correction Factors—*

Folin<sup>3</sup> recommended that 2 per cent should be subtracted from the values obtained for the chemical estimation of total acid soluble and inorganic phosphate of the plasma in order to correct for the volume occupied by the protein precipitate. This correction was applied to both the chemical determinations and to the counting rates observed for these two plasma fractions.

It was determined experimentally that the protein from red blood cells occupied approximately one tenth of the total volume in the dilution used. Accordingly, 10 per cent was subtracted from the chemical results and also from the counting rates obtained for total acid soluble and inorganic phosphorus in the red blood cells.

As pointed out earlier additional corrections were necessary for some of the radioactive preparations because of errors in counting introduced by the physical characteristics and geometry of the dried samples. In addition all results obtained with a Geiger Muller counter and Auto scaler are subject to certain intrinsic instrumental inaccuracies.

Dilutions of aqueous solutions of radioactive sodium phosphate were prepared from which duplicate 2 ml samples were pipetted into the same type of bottle caps used in all subsequent experiments. After the samples were thoroughly dried each of the duplicates was counted three times. In the first column of Table I are given the means of the six readings taken on each of the fifty samples. The standard deviation from the mean of the entire series, after correcting for background was approximately  $\pm 3$  per cent.

TABLE II EFFECT OF VARYING AMOUNTS OF ALBUMIN ON  $P^{32}$  COUNTING RATE

WT OF ALBUMIN SAMPLE (MG PER CM <sup>2</sup> )	PER CENT OF COUNTS LOST
5.2	0
8.2	0
10.5	0
13.1	3
15.9	4
18.5	5
21.0	7
27.3	9
40.4	15
52.9	18
66.7	23
79.6	26
92.4	30
105.0	34

All figures are based on the average of duplicate determinations, three observations being taken on each of the duplicate.

TABLE III EFFECT OF VARYING AMOUNTS OF TRICHLOROACETIC ACID ON  $P^{32}$  COUNTING RATE

WT OF TRICHLOROACETIC ACID SAMPLE (MG PER CM <sup>2</sup> )	PER CENT OF COUNTS LOST
1.3	0
1.7	0
3.4	0
5.2	1
8.5	2
11.3	2
14.0	5
16.9	2
17.8	3
18.2	6
23.7	5
24.4	6
27.0	15
29.8	9
37.5	18
44.0	24
52.7	27
71.5	30

All figures are based on the average of duplicate determinations, three observations being taken on each of the duplicates.

Additional errors, most likely introduced by the thickness and uneven surfaces of the dried samples, were present. Kamen<sup>2</sup> has stated that the beta rays from  $P^{32}$  may show significant absorption due to thickness of the sample film when the weight of the sample exceeds 15 to 20 mg per square centimeter. Table II shows the corrections necessary when known amounts of  $P^{32}$  were added to varying concentrations of human serum albumin. The samples were measured into weighed bottle caps, thoroughly dried, and reweighed. From physical measurements the area of the bottle caps was estimated to be 4.9 cm<sup>2</sup>, and the milligrams per square centimeter of albumin were calculated for each sample. In Table III correction factors derived from a similar experiment with trichloroacetic acid are given. In both cases, as expected, the counting rate decreased as the thickness of the dried films increased. These results will be discussed later.

## RESULTS

*Total  $P^{32}$  in Plasma*—To determine the correction factor which should be applied to the results obtained for total  $P^{32}$  in plasma, a series of twenty samples was prepared containing plasma to which known amounts of  $P^{32}$  were added in vitro. The dilution necessarily introduced by the addition of  $P^{32}$  solution was kept as small as possible and never exceeded 2 per cent of the total volume. The readings were compared with those of 2 ml amounts of aqueous solutions containing the same concentrations of  $P^{32}$  as the plasma preparations. Each sample was counted at least three times. The results obtained by counting the emissions from the aqueous preparations were regarded as 100 per cent recovery. Statistical analysis of the results showed the average recovery in the plasma samples to be 89 per cent with a standard deviation of  $\pm 3.95$  (Table IV). On the basis of the recovery experiments, a correction factor of +11 per cent was applied to

TABLE IV RECOVERY OF  $P^{32}$  IN PLASMA  
(1.98 ML OF PLASMA IN 2 ML SAMPLES, 0.02 ML AQUEOUS  $P^{32}$  SOLUTION)

PER CENT RECOVERY*	PER CENT RECOVERY
87	86
87	93
92	93
92	81
91	82
87	90
86	91
89	91
90	89
92	98
Mean recovery = 89 per cent	
Standard deviation = $\pm 3.95$	

\*Each figure is based on the average of at least three observations.

all counts observed for total  $P^{32}$  in plasma. Two milliliters of normal human plasma when dried were found to weigh on an average about 34 mg per square centimeter. Table II shows a loss of 12 per cent of added  $P^{32}$  in the presence of albumin of equivalent weight, which agrees closely with the recovery experiments presented in Table IV.

*Total  $P^{32}$  in Red Blood Cells*—The weight of 2 ml of a 1:5 dilution of red blood corpuscles was found to be approximately 21 mg per square centimeter. From Table II it might be predicted that about 94 per cent of the radioactivity



present would be detected. To study this point more thoroughly, twenty eight *in vitro* recovery experiments were performed in which 2 ml samples of red blood cells diluted 1:5 and containing known concentrations of  $P^{32}$  were dried and counted. Each sample was counted at least three times. The average recovery of the added  $P^{32}$  was 95 per cent (standard deviation of  $\pm 3.9$ ) of that observed for dried aqueous solutions containing the same concentrations of  $P^{32}$  (Table V). This is in excellent agreement with the expected recovery. Consequently, a correction of 5 per cent was applied to the observed counts for total  $P^{32}$  in red blood cells.

TABLE V RECOVERY OF  $P^{32}$  ADDED TO RED BLOOD CORPUSCLES DILUTED 1:5 WITH DISTILLED WATER

PER CENT RECOVERY*	PER CENT RECOVERY
99	93
99	90
98	96
93	90
94	94
95	93
92	95
90	98
92	97
90	98
96	99
105	99
88	96
89	94
Mean recovery = 95 per cent	
Standard deviation = $\pm 3.9$	

Each figure is based on the average of at least three observations

*$P^{32}$  in the Total Acid-Soluble Phosphate Fraction of Plasma*—To determine the necessary correction factor for the measurements of  $P^{32}$  in the total acid soluble phosphate fraction of plasma, thirty-two samples were prepared containing known amounts of  $P^{32}$  and 0.16 Gm of trichloroacetic acid in 2 milliliters. This concentration of trichloroacetic acid was the same as that of the plasma filtrates. Each sample was counted at least three times. The average recovery was found to be 83 per cent with a standard deviation of  $\pm 7.48$  as compared with aqueous dilutions containing the same amount of  $P^{32}$  (Table VI). Two milliliter samples of the trichloroacetic acid filtrate prepared from plasma were found to weigh about 37 mg per square centimeter. Table III indicated that a recovery of 82 per cent of the added  $P^{32}$  would have been expected on the basis of film thickness. Thus the observed mean recovery agrees well with this value. The fairly large standard deviation is probably due to the very considerable physical differences in the dried trichloroacetic acid film. Modifications of this part of the procedure are proceeding.

A series of experiments was performed which indicated that filtration through Whatman No. 42 paper did not contribute to losses in radio activity. Consequently the observed counts for the total acid soluble fraction of plasma were corrected on the basis of the *in vitro* recovery experiments presented in Table VI which showed an average loss of 17 per cent of added  $P^{32}$ , together

TABLE VI RECOVERY OF  $P^{32}$  IN TRICHLORACETIC ACID, 0.16 GM TRICHLORACETIC ACID IN 2 ML

(COPRESPONDS TO PLASMA TOTAL ACID SOLUBLE FRACTION)

PER CENT RECOVERY*	PER CENT RECOVERY
73	84
69	84
85	83
85	78
84	87
64	97
64	78
84	84
83	92
82	84
83	82
82	84
83	89
83	92
83	94
82	94
Mean recovery = 83 per cent	
Standard deviation = $\pm 7.48$	

\*Each figure is based on the average of at least three observations

with the correction of 2 per cent to compensate for the volume of the protein precipitate. Hence, 15 per cent was added to all counts observed for total acid-soluble phosphate in plasma.

*$P^{32}$  in the Total Acid-Soluble Phosphate Fraction of Red Blood Cells—*

Two milliliter samples obtained from red blood cell protein free filtrates contained 0.08 Gm of trichloroacetic acid and were found to weigh approximately 17 mg per square centimeter. Table III shows a recovery of about 97 per cent for samples of this weight. Eighteen recovery experiments containing known amounts of  $P^{32}$  and 0.08 Gm of trichloroacetic acid in 2 ml amounts were counted and found to have an average of 97 per cent recovery of the added  $P^{32}$  with a standard deviation of  $\pm 6.1$  (Table VII). Again, the fairly large standard deviation is probably accounted for by differences in the physical characteristics of the dried trichloroacetic acid film. Since no counts were considered to be more accurate than  $\pm 3$  per cent, no corrections were made for absorption due to thickness of film, but 10 per cent was subtracted from all counts observed in the total acid soluble phosphate fraction of red blood cells to correct for the volume occupied by protein.

TABLE VII RECOVERY OF  $P^{32}$  IN TRICHLORACETIC ACID, 0.08 GM TRICHLORACETIC ACID IN 2 ML

(CORRESPONDS TO RED BLOOD CELL TOTAL ACID SOLUBLE FRACTION)

PER CENT RECOVERY*	PER CENT RECOVERY
110	104
108	100
94	98
93	95
93	90
89	94
100	92
102	92
102	95
Mean recovery = 97 per cent	
Standard deviation = $\pm 6.12$	

\*Each figure is based on the average of at least three observations

$P^{32}$  in the Inorganic Phosphate Fraction of Plasma and Red Blood Cells—No corrections for loss of  $P^{32}$  by selfabsorption were applied to samples of inorganic phosphate. These samples when dried weighed about 1 mg per square centimeter. Sixteen recovery experiments showed an average recovery of 98 per cent of added  $P^{32}$  with a standard deviation of  $\pm 4.2$  (Table VIII). Two per cent was subtracted from the observed counting rates for plasma to correct for the volume occupied by protein. For the same reason 10 per cent was subtracted from results obtained for red blood corpuscles.

TABLE VIII RECOVERY OF  $P^3$  IN INORGANIC PHOSPHATE FRACTIONS

PER CENT RECOVERY	
PLASMA	RED BLOOD CELLS
95	103
95	94
90	96
92	101
95	97
94	96
	105
	106
	100
	98
Mean recovery = 98 per cent	
Standard deviation = $\pm 4.2$	

Each figure is based on the average of at least three observations.

$P^{32}$  in Urine—Urine samples appeared to offer no difficulties from the standpoint of counting techniques. Their thickness and consistency did not differ enough from that of the control aqueous solutions of  $P^3$  as sodium phosphate to introduce any significant errors. All of the *in vitro* tests performed in which known amounts of  $P^3$  were added to urine indicated 97 to 103 per cent recovery of the added  $P^{32}$ . Some modification of the method might be necessary for urine containing large amounts of protein but such complications have not been encountered.

It is apparent from the data presented that numerous corrections principally for selfabsorption, are required in order to make reliable determinations of  $P^{32}$  in plasma and red cells by beta particle counting. The correction factors necessary may be determined experimentally as in the present communication, or selfabsorption correction factors may be approximated from the following equation presented in a simplified theory in an article by Fluharty<sup>6</sup>

$$\frac{N}{N_0} = \frac{1}{\mu g} (1 - e^{-\mu g})$$

where  $N_0$  is the counting rate without absorber,  $N$  is the counting rate with  $g$  grams per square centimeter of absorber, and  $\mu$  is the mass absorption coefficient.

The relation between  $\mu$  and the maximum beta ray energy is given roughly ( $E_{\max} > 0.5$  mev) by

$$\mu = 22/E_{\max}^{4/3}$$

where  $E_{\max}$  is the maximum energy of the beta rays emitted.  $E_{\max}$  is 1.72 mev and  $\mu$  is 10.7 for  $P^{32}$ .

Fig 1 presents a plot of the theoretical ratio  $\frac{N}{N_0}$  as a function of milligrams of sample in each square centimeter assuming that the total activity is contained in each sample, but uniformly distributed throughout. Also plotted are the observed values as found experimentally. It may be seen that the

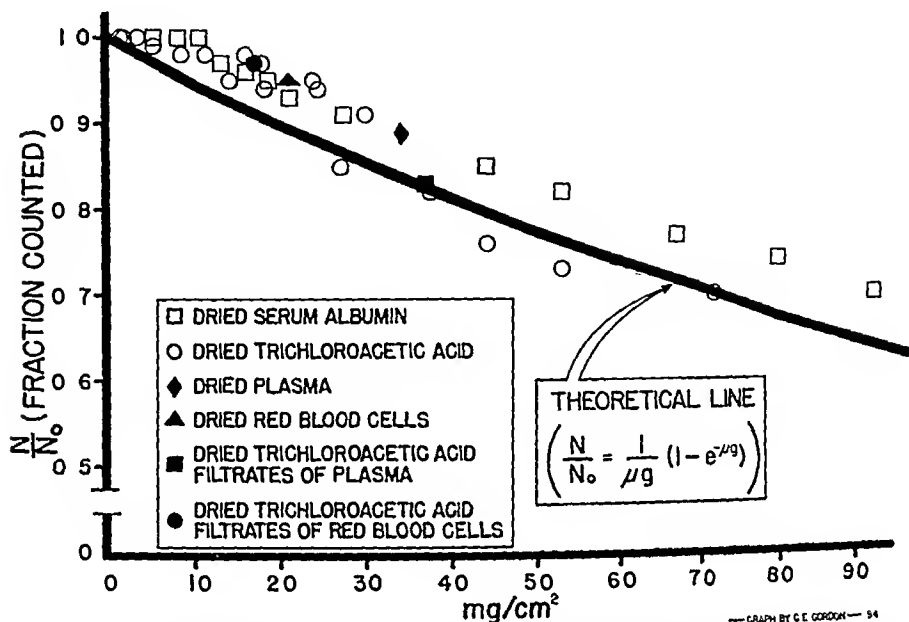


Fig 1—Relationship between ratio of observed and true counts and milligrams per square centimeter sample counted. Solid line represents the theoretical counts based on the relationship between beta ray energy and milligrams per square centimeter as shown in the equation on the chart.

agreement between theory and experiment is good to within 5 per cent. Except for isolated instances, the fraction of counts observed was greater than the experimental value, but the shape appears proper aside from low quantities of absorbing materials. The discrepancy at low grams per square centimeter is not unexpected, although a satisfactory explanation with experimental proof has not been given. Most likely the variation from theory represents low readings in the standard due to piling up on the edges and subsequent poor geometry with respect to the Geiger tube. The spread in value can be attributed to the differences in the physical character of the different samples.

#### SUMMARY AND CONCLUSIONS

1 Methods are described for the determination of radioactive phosphorus in plasma, red blood corpuscles, and urine following the intravenous injection of 100 to 200 microcuries of the isotope in normal human subjects. These methods also are applicable to *in vitro* experiments.

2 The separation of the total acid-soluble and inorganic phosphate fractions of plasma and red blood corpuscles is described and the procedures used for preparing and counting radioactive samples from these fractions are given.

3 Losses of radioactivity by selfabsorption in thick samples are discussed

4 Certain correction factors are necessary for comparison of counting rates obtained in samples possessing different physical characteristics The derivation and use of such correction factors are discussed

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# A METHOD FOR DETERMINING $\alpha$ -AMYLASE ACTIVITY

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UNTIL recently, methods for determining  $\alpha$ -amylase activity in serum or urine were based on the estimation of the reducing sugars formed during the enzymic hydrolysis of starch. From the work of Cattle and Hanes<sup>1</sup> it became apparent that the rate of starch hydrolysis could be followed by determining the decrease in starch concentration as measured by the color of the starch-iodine complex. During the last year, while the present work was in progress, two communications appeared<sup>2, 3</sup> which describe methods based on this principle. It is clear from these investigations, and from our own, that  $\alpha$ -amylase initially catalyzes the production of starch fragments which yield a different color (or none) with iodine than does starch, that the decrease in starch concentration, as evidenced by the starch-iodine complex color, can be estimated accurately photometrically, and that the decrease in starch concentration initially is a measure of  $\alpha$ -amylase activity. Because of the appearance of the latter publications<sup>2, 3</sup> our own experiments concerning the basic principles will not be reported. This paper will be limited to a description of the method developed which seems somewhat simpler than those previously described and appears to be quite suitable for routine work.

## METHOD

*Reagents*—1 Starch solution 0.1000 Gm of Merck soluble starch and 0.3 Gm NaCl were dissolved in 250 ml 0.1M Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.0. This solution cannot be stored for more than one or two days.

2 Iodine solution 1.27 Gm iodine and 10 Gm KI were dissolved up to 1,000 ml with distilled water. If stored in a glass stoppered bottle the solution is stable for about one month.

*Procedure*—To numbered Klett tubes containing 3.0 ml ice cold starch solution are added 0.5 ml aliquots of whole serum or serum diluted with normal saline. A blank was included. The contents of the tubes are quickly mixed and incubated in a 37° C water bath for exactly five minutes and then placed in an ice water bath. Using the same order of addition, 3.0 ml of the iodine reagent are added immediately and rapidly from a burette. After several minutes, 4.0 ml distilled water are added and the contents well mixed. In exactly fifteen minutes from the time of addition of iodine reagent the color is read with the Klett Summerson photoelectric colorimeter using filter 66. It should be noted that the iodine reagent slows amylase action markedly but does not abolish it completely.

## RESULTS

To assay the sensitivity of the method and linearity of response, the following experiment was performed. Aliquots of a normal serum were diluted with 0.9 per cent saline as indicated in Table I and the  $\alpha$ -amylase activity was determined as described in the previous section. The results indicate that as little as 0.1 ml of serum can be used and that there is a linear relationship between enzyme activity and starch-iodine color.

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TABLE I  $\alpha$  AMYLASE ACTIVITY OF DILUTED SERUM

ML. WHOLE SERUM PER 0.5 ML ALIQUOT OF DILUTED SERUM	OPTICAL DENSITY $\times 1000$
0	350
0.100	256
0.250	192
0.333	164
0.400	135
0.500	104

If it is desirable enzyme activity can be expressed in units. One unit is defined as the amount of enzyme in 10 ml which causes the hydrolysis of 0.01 mg of starch in one minute at 37° C. in a phosphate buffer of pH 7.0. Therefore,

$$\frac{12 \left(1 - \frac{A}{B}\right)}{0.01 \times 5 \times 0.5} = \text{units}$$

where A is the density reading of the unknown, B is the density reading of the blank, 12 is the number of milligrams of starch in 30 ml of substrate, 0.01 is the number of milligrams of starch hydrolyzed per unit activity per minute, 5 minutes is the duration of incubation and 0.5 is the number of milliliters enzyme solution taken.

TABLE II REPRODUCIBILITY OF  $\alpha$  AMYLASE ASSAY METHOD

SAMPLE	INEXPERIENCED STUDENT		EXPERIENCED STUDENT	
	DENSITY $\times$ 1,000	UNITS	DENSITY $\times$ 1,000	UNITS
A	151	21.5	150	27.3
	149	27.5	149	27.5
	149	27.5	153	27.0
B	111	32.8	109	33.0
	103	33.9	104	33.8
	104	33.8	102	34.0
Blank	350		350	

In order to judge reproducibility, the results of a student performing the test for the first time were compared with those obtained by one having experience with the method. The data are summarized in Table II. The time required for the analysis of six samples is approximately thirty minutes.

Sera from several students selected at random were assayed for  $\alpha$  amylase activity. Table III indicates the range of normal values obtained.

TABLE III  $\alpha$  AMYLASE ACTIVITY OF NORMAL SERA

SUBJECT	AGE	ACTIVITY IN UNITS
Male	28	27.3
Female	23	33.6
Male	26	32.8
Male	24	28.8
Male	27	28.4

## SUMMARY

A simple photometric method for determining  $\alpha$ -amylase activity has been developed which is applicable as a routine method in the clinical laboratory

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# A SIMPLE, INEXPENSIVE APPARATUS FOR THE DESICCATION OF BACTERIA AND OTHER SUBSTANCES

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THE advantages of desiccation from the frozen state have been mentioned many times in the literature. The principles involved will not be detailed here, as the reader may find these in many excellent reviews such as those of Greaves<sup>1</sup> and Flosdorf and co workers.

The apparatus to be described has the following advantages (1) the device is simply and easily constructed, (2) the cost of operation is low, (3) it is versatile. Material may be dried at various temperatures and to a definite moisture content. The dried material may be sealed off *in vacuo* or under various gases.

## CONSTRUCTION

The apparatus as seen in the photograph Fig 1 is quite simple. It consists of a Pyrex cylinder about 2 in in diameter supported horizontally and of a length to suit the convenience of the operator. It is closed at both ends and has a ground glass stopper on the top. Five or more side arms of  $\frac{1}{4}$  in glass tubing extend out on each side at about 10 and 2 o'clock to which are fastened by means of rubber tubing the F tubes and ampules. The cylinder will conveniently hold 100 cc of sulfuric acid which is the drying agent.

## OPERATION

The sulfuric acid is placed in the apparatus with the aid of a funnel. The stopper is greased with silicone stopcock grease and replaced. The F tubes, ampules, and rubber tubing are also lightly coated with silicone grease and placed on the side arms. The small U tube mercury manometer is placed on the special arm at one end and the vacuum pump connected to the other. The ampules are frozen in a bath of dry ice (solid carbon dioxide) and alcohol and the vacuum pump is turned on. When a vacuum of 0.5 mm or more is obtained, the dry ice and alcohol bath is removed and the material allowed to dry. The liquid in the ampules may be "snap frozen" if dry ice is not available. This is done by cautiously opening the connection between the pump and the machine. Air in solution in the material to be dried will be evolved as bubbles. When all this air is expelled the connection between the pump and the machine may then be opened completely. The contents of the ampules then will rapidly fall in temperature and within a few minutes will suddenly freeze. The size of the ampule should be large in proportion to the amount of liquid to prevent bubbles being drawn into the neck of the ampule. After a vacuum has been obtained,

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line The next line indicates the temperatures of a condenser which in a closed system will produce these vapor pressures The next seven lines indicate the percentage by weight of sulfuric acid necessary at several temperatures to produce these pressures On the lower left are indicated the final temperatures of the dried milk

If one wishes to dry milk to a moisture content of, for example, 2.5 per cent at room temperature ( $25^{\circ}\text{C}$ ), one chooses a bar which is the same length as the distance from 0 of the moisture scale to the 2.5 mark This bar should

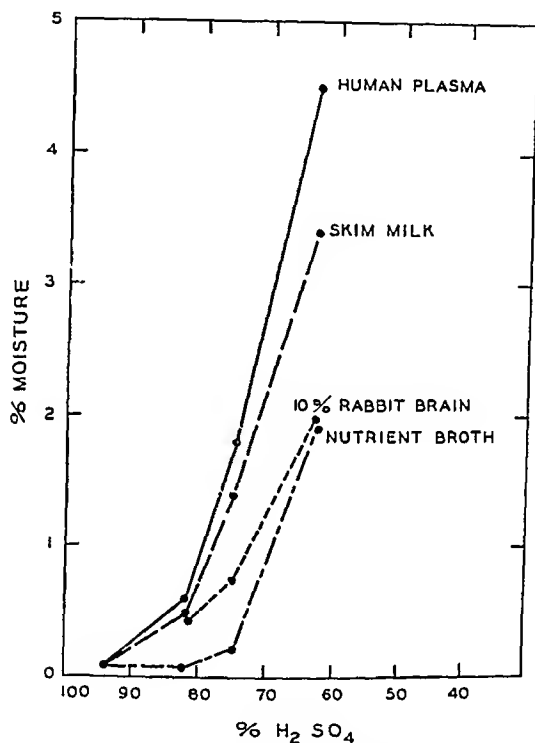


Fig. 3—Relationship between moisture content of various substances and vapor pressure at equilibrium (three days at  $35^{\circ}\text{C}$ )

rest on the  $25^{\circ}\text{C}$  line Following vertically upward from the base of this bar, we find a vapor tension of 12 mm of Hg On this same line, we find  $-15^{\circ}\text{C}$ , which is the temperature of a condenser giving this vapor pressure If we wish to use sulfuric acid as a desiccant at room temperature, we find that approximately 69 per cent will be right

In Fig. 2 the moisture content of milk was determined by placing weighing bottles of dried milk in desiccators over various concentrations of sulfuric acid until the weight remained constant They were then held over  $\text{P}_2\text{O}_5$  under a vacuum to constant weight The loss of weight over  $\text{P}_2\text{O}_5$  was considered a water loss Knowing the weight of the sample, the moisture content was calculated The other data included were obtained from standard tables <sup>4, 5</sup>

The chart (Fig 2) was based on skim milk as we have found this a suitable vehicle for drying organisms. The data in Fig 2 will not apply when predicting the moisture content of other substances dried over sulfuric acid.

As shown in Fig 3, nutrient broth, skim milk, rabbit brain, and human plasma have been dried for three days over several concentrations of sulfuric acid. Each concentration of sulfuric acid will produce a different water vapor

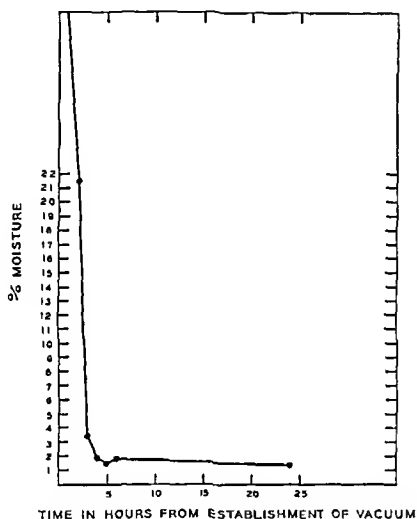


Fig 4.—Relation between moisture content of milk and time of desiccation on the apparatus described. Moisture contents determined with Fischer's agent. Irregularity at 6 hours probably due to absorption of water into ampule which was removed in the evening and tested the next morning.

tension. It is apparent that for any given vapor tension the moisture content of one substance may differ from the moisture content of another.

Desiccation with this apparatus is quite rapid, as illustrated in Fig 4. It can be seen that equilibrium has been reached in five hours.

Using *Vibrio cholerae* as a test organism, drying runs were made on this apparatus and on another manifold type of apparatus which employed dry ice and Methylcellosolve for moisture removal. On three runs using sulfuric acid, the percentage of organisms remaining viable after drying, as determined by plate counts done before and after drying, was 13 per cent, 0.05 per cent, and 0.5 per cent. The percentage of viable organisms on two runs with dry ice was 0.4 per cent and 0.05 per cent.

## SUMMARY

An apparatus is described for desiccation of biologicals and other substances which is cheap in initial and operational cost and which will dry to a pre-determined moisture content. A graph is presented showing the relations between the moisture content of milk, sulfuric acid concentration, condenser temperature, and vapor tension.

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ERRATUM

In the article by Innes, Innes, and Moore, "The Hematologic Changes Induced in Guinea Pigs by the Prolonged Administration of Pteroyl Glutamic Acid Antagonists," in the July issue of the *JOURNAL*, Met Fol B was stated to be 7-methyl pteronic acid. That is incorrect. Met Fol B is N<sup>10</sup>-methyl pteronic acid. In the list on p. 885, therefore, and throughout the article, please read N<sup>10</sup>-methyl pteronic acid instead of 7-methyl pteronic acid.

# BLOOD COAGULATION IN LEUCEMIA AND POLYCYTHEMIA VALUE OF THE HEPARIN CLOTTING TIME AND CLOT RETRACTION RATE

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## INTRODUCTION

THROMBOSIS and hemorrhage are two of the most prominent causes of complication and morbidity in disease. This is particularly true of leukemia and polycythemia vera. Thrombosis especially of the cerebral and coronary vessels, represents the most serious threat to life in polycythemia<sup>1 2 4</sup>. Curiously enough hemorrhage also occurs in this disease. It is a frequent symptom in the form of epistaxis and bleeding gums or may be quite severe after any type of surgical operation even tooth extraction<sup>6 7</sup>. In leukemia hemorrhage has long been a widely recognized finding and a therapeutic problem<sup>1 8</sup>.

The value of studying the blood coagulation mechanisms in these diseases is readily apparent. However a review of the literature revealed no adequate or extensive study of blood coagulation in these conditions except for a study on thrombocytopenic purpura in leukemia by Rosenthal<sup>9</sup>.

In acute leucemia and in many cases of chronic leucemia bleeding can be attributed to a marked depression in platelet count and function. In these instances, the usual tests for platelet function such as bleeding time, tourniquet test and presence of clot retraction give abnormal results. Bleeding in the absence of a definite thrombocytopenia or abnormality of the above mentioned clotting or hemostatic tests as is frequently observed in chronic leucemia has never been explained adequately. In his monograph on leukemia Forkner<sup>10</sup> stated: "Frequently no adequate explanation can be advanced as to the cause of bleeding in leukemia. The problem has been studied inadequately."

The general opinion upon coagulation, thrombosis and hemorrhage in polycythemia vera has been well summarized by Harrop and Wintrobe<sup>11</sup>:

"The blood platelets may be normal in number but are frequently increased sometimes to three or four times their normal values. The various tests of the efficiency of blood coagulation reveal no striking abnormality. Bleeding time is usually essentially normal and coagulation time normal. In spite of thrombocytosis clot retraction may be slow and little serum is usually formed probably because of the great increase in the size of the blood mass. These features may explain not only the abnormal tendency to thrombosis in this disease but the blood vessel engorgement and slow clot retraction suggest an explanation for the frequent hemorrhages."

From the Division of Medical Physics and the Donner Laboratory, University of California. This work was supported in part by a grant from the United States Public Health Service.

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It also has been observed that the clot forming from polycythemic blood is fragile and may dissolve or become fluid upon minimal agitation<sup>12</sup>

It is the purpose of this paper to report the results of a one and one half year study of blood coagulation in forty-five patients with polycythemia vera, twenty-eight patients with leucemia and sixteen patients with other diseases. During the course of this study, two new tests of blood coagulation were developed and carefully standardized upon sixty-four normal subjects. These tests are

1 Clot retraction rate A quantitative measure of clot retraction obtained by electric resistance measurements

2 Heparin clotting time A modification and simplification of the heparin tolerance test, which measures the effect of added heparin upon the clotting time of blood

### METHODS

All blood coagulation tests were performed in a water bath regulated at 37° C upon venous blood carefully drawn to avoid tissue juice contamination. A dry syringe and 20 gauge needle were used. The first and last cubic centimeters of blood in the syringe were not used for these tests. Blood counts were performed upon capillary blood.

*Clot Retraction Rate*—A method\* was developed and described by Rosenthal and Tobias<sup>13</sup> by which a quantitative measure of clot retraction, as well as other information, may be obtained by the measurement of the electric resistance of freshly drawn blood, as it clots. Fig 1 shows a typical electric resistance curve with explanations and illustrations of the changes during coagulation.

Since the increase in electric resistance is caused by retraction of the clot around the electrodes, the slope of the rising portion of the resistance curve is comparable to the "clot retraction rate." The resistance increase was usually linear and most rapid during the first forty minutes of measurement. This period also was subject to the least amount of error and variation such as result from the edge of the electrode cutting through the retracting clot. The clot retraction rate is calculated in the following manner:

$$\text{Clot retraction rate in ohm centimeters per minute} = \frac{\text{Specific resistance at 35 min} - \text{Specific resistance at 15 min}}{35 \text{ min} - 15 \text{ min}}$$

Normal range 4 to 10 ohm cm per minute, average value 7.1 ohm cm per minute

*Heparin Clotting Time or Heparin Prolonged Clotting Time*—This test measures the effect upon the clotting time of the addition of 0.04 mg of heparin to 1 cc of venous blood. The use of this amount of heparin was arrived at by an extensive study upon the effects of various amounts of heparin upon the clotting time of blood.

Pyrax test tubes, 100 mm by 13 mm in dimensions, were marked with a scratch at a level coincident with the bottom part of the meniscus of 11 cc of fluid. Heparin† was diluted to provide 0.04 mg per 0.1 cc of isotonic saline, 0.1 cc of this heparin solution was placed in a dry test tube prepared as described. The clotting time was then determined after placing freshly drawn blood in the tube up to the scratch mark, thereby adding 1 cc. of blood. The blood and heparin were thoroughly mixed by inverting the tube twice. A stopper was inserted and the tube was examined with minimal tilting and agitation for the formation

\*The method of electric resistance measurement was revised by Dr C W Tobias assisted by Mr L Lipetz. Measurements since June 1948 have been obtained by comparing the potential across the electrodes to a standard potential. A constant current is used and the resultant potential across the electrodes is directly related to the electric resistance. Measurements are recorded automatically by a 6 channel Speedomax (Leeds and Northrup) every twelve seconds for each sample.

†Heparin made by Lederle Laboratories was used in this study. Most commercial preparations which may be used in this test consist of the purified sodium salt of heparin in solution 1 cc. containing 10 mg of heparin.

of a firm clot at one minute intervals beginning at twelve minutes. The end point or clotting time occurred when the tube could be completely inverted without any fluid breaking from the clot. Careful attention to three factors in the technique eliminated much error and variation. 1 Precise measurement of the volumes of heparin solution and blood. The use of a

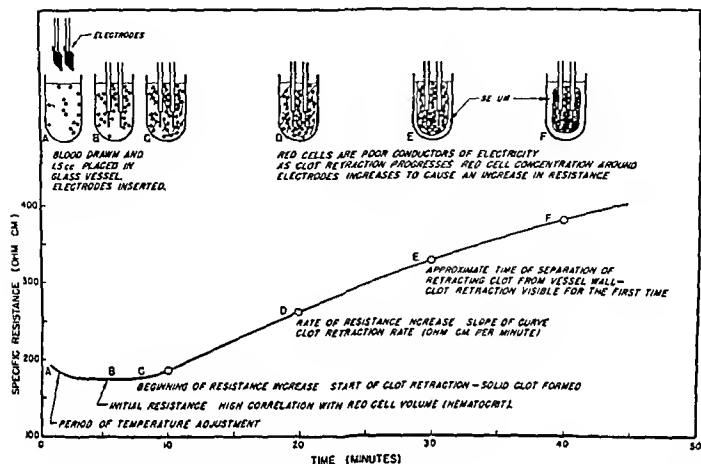


Fig 1—Typical electric resistance curve with explanations and illustrations of the changes during conglutination

pipette for the measure of blood was not necessary if the relation between the scratch mark and meniscus of the blood was consistent. 2 Thorough mixing of the heparin with the blood. 3 Minimal agitation of the blood in the determination of the clotting time. In the course of this study it was possible to obtain good end points for clotting times as long as five hours. Duplicate measurements upon aliquot samples of blood showed an average variation of less than 5 per cent.

Normal range 20 to 35 minutes. Average value 27.5 minutes.

**Clotting Time (Lee White)**—This is a variation of the Leo White clotting time. One cubic centimeter of blood was placed in a dry Pyrex tube, 100 mm by 13 mm. in dimensions. The tube was examined for clot formation by minimal tilting every half minute. Normal range 5 to 10.5 minutes.

**Toluidine Blue Titration Test**—This test measured the effect of a series of concentrations of toluidine blue on the clotting time of recalcified oxalated plasma. Venous blood was mixed with 0.1 molar sodium oxalate nine parts to one and centrifuged for fifteen minutes at medium speed in a small clinical centrifuge. One tenth cubic centimeter of plasma was added to each of six increasing amounts of toluidine blue\* in 0.05 cc of isotonic saline. One tenth cubic centimeter of 0.25 molar calcium chloride solution was added to each tube which was then examined every fifteen seconds for clot formation. A control tube containing saline without toluidine blue was also measured.

**Clot Retraction by Inspection**—The amount of clot retraction was estimated by external inspection of the clots formed in the electric resistance, heparin clotting, and Lee White clotting tubes at four and twenty four hours.

\*Toluidine blue O made by National Aniline Division Allied Chemical and Dye Corp. New York N. Y. Total dyo content was 69 per cent.

*Bleeding Time*—The method described by Duke<sup>14</sup> was used. The ear lobe was firmly punctured with a pointed scalpel blade and was observed every half minute for cessation of bleeding. Normal range 2.5 to 6 minutes.

*Prothrombin Time*—A variation of the Quick<sup>15</sup> method described by Rosenfield and Tuft<sup>16</sup> was used with a few modifications. Dried rabbit brain thromboplastin (made by Difco Laboratories) was freshly prepared each time measurements were made. 0.125 molar calcium chloride was used. Determinations were made on 100 per cent and 10 per cent plasma, which was diluted with barium sulfate treated plasma. Normal ranges 100 per cent plasma, 14.0 to 18.0 seconds, 10 per cent plasma, 24.0 to 35.0 seconds.

*Tourniquet Test*—A rubber tourniquet was placed snugly around the lower part of the arm for five minutes without obliterating the arterial pulse. A positive test consisted of the appearance of numerous petechiae below the level of the tourniquet.

*Hematocrit*—Heparinized blood was placed in a Wintrobe tube and centrifuged for one half hour at 2,000 revolutions per minute. The reading of the red cell volume in per cent was then made. Normal range 40.5 to 51.0 per cent.

*Sedimentation Rate of Red Cells*—Heparinized blood was placed in a Wintrobe tube and the amount of sedimentation of the red cells was measured in millimeters after one hour.

*Platelet Count*—Red cell pipette and Rees Eekers diluting solution were used. Counts were determined upon capillary blood. Normal range 200,000 to 400,000.

Red and white cell counts and differentials were performed according to routine laboratory technique. A photoelectric method was used for the hemoglobin determinations.

#### MATERIAL AND PROCEDURE OF STUDY

The coagulation and hematologic studies were performed both upon normal, apparently healthy subjects and upon patients seen at the Donner clinic. All the patients were ambulatory and were distributed from a standpoint of diagnosis as follows: polycythemia vera—untreated, thirty nine, treated (in remission), four, leucemic phase, two, leucemia—acute, two, chronic myelocytic, nine, chronic lymphocytic, sixteen, chronic eosinophilic (?), one, lymphoma, four, multiple myeloma, two, agnogenic myeloid metaplasia, two, secondary polycythemia, two, other diseases (nonhematologic), six.

At various stages of the study, different groups of coagulation tests were performed. In the last two thirds of the study the following tests were routinely performed: clot retraction rate (electric resistance measurement of the blood), heparin clotting time, Lee White clotting time, hematocrit, and platelet count. Bleeding time, tourniquet test, erythrocyte sedimentation rate, and toluidine blue titration were performed on selected patients. The prothrombin time was employed only in the early stages of the study.

Many of the patients have been studied at various intervals in order to relate the coagulation picture to the hematologic and clinical course and the effect of radioactive isotopes and other therapy. However, this report is primarily devoted to the consideration of the initial studies for each patient.

#### RESULTS

The results for each test will be described briefly and then some significant relationships among the coagulation tests, hematologic picture, and clinical status will be presented.

*Clot Retraction Rate*—Fig. 2 shows the results of this test for the normal subjects and various groups of patients. For the normal subjects, the presence of two peaks can be seen at 6 to 7 and 8 to 9 ohm cm per minute. The first range corresponds to the peak of distribution of seventeen female subjects, the second to that of thirty-six male subjects. The average values of each of these groups were not statistically different. Eleven of twenty-five leucemic patients showed clot retraction rates below the lower limit of normal, while twelve of



forty one patients with polycythemia vera had values above the normal range, indicative of increased clot retraction. The distributions for the other groups are apparent.

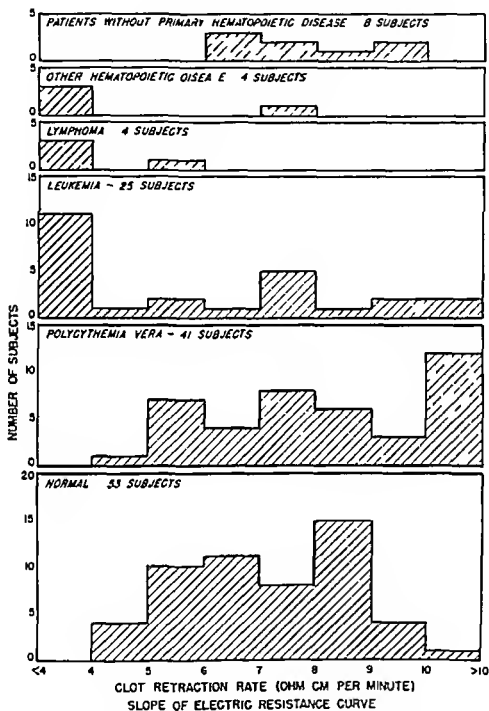


Fig. —Distribution of results of the clot retraction rate for the various groups of subjects.

The relative effect of various factors involved in clot retraction upon the clot retraction rate was indicated by further analysis of the data and examination of the pattern of the electric resistance curves. The platelet count appeared to be the most important factor showing a high correlation coefficient with the clot retraction rate of +.83. The hematocrit as indicated by the initial blood resistance had only a negligible effect upon the clot retraction rate. The presence of an abnormally rapid red cell sedimentation rate showed its effect upon the pattern of the electric resistance curves as illustrated and explained in Figs. 3, 1 and B. This unusual pattern called the 'atypical electric resistance curve' usually occurred in the blood of leucemic patients with low hematocrits and rapid red cell sedimentation rates. The results indicate how

ever, that the clot retraction rates computed from these curves are not significantly altered by the red cell sedimentation. Fig 3, C and D, gives examples of resistance curves giving low and high clot retraction rates respectively. Twelve duplicate measurements on aliquot samples of blood showed an average variation in clot retraction rate of 10 per cent.

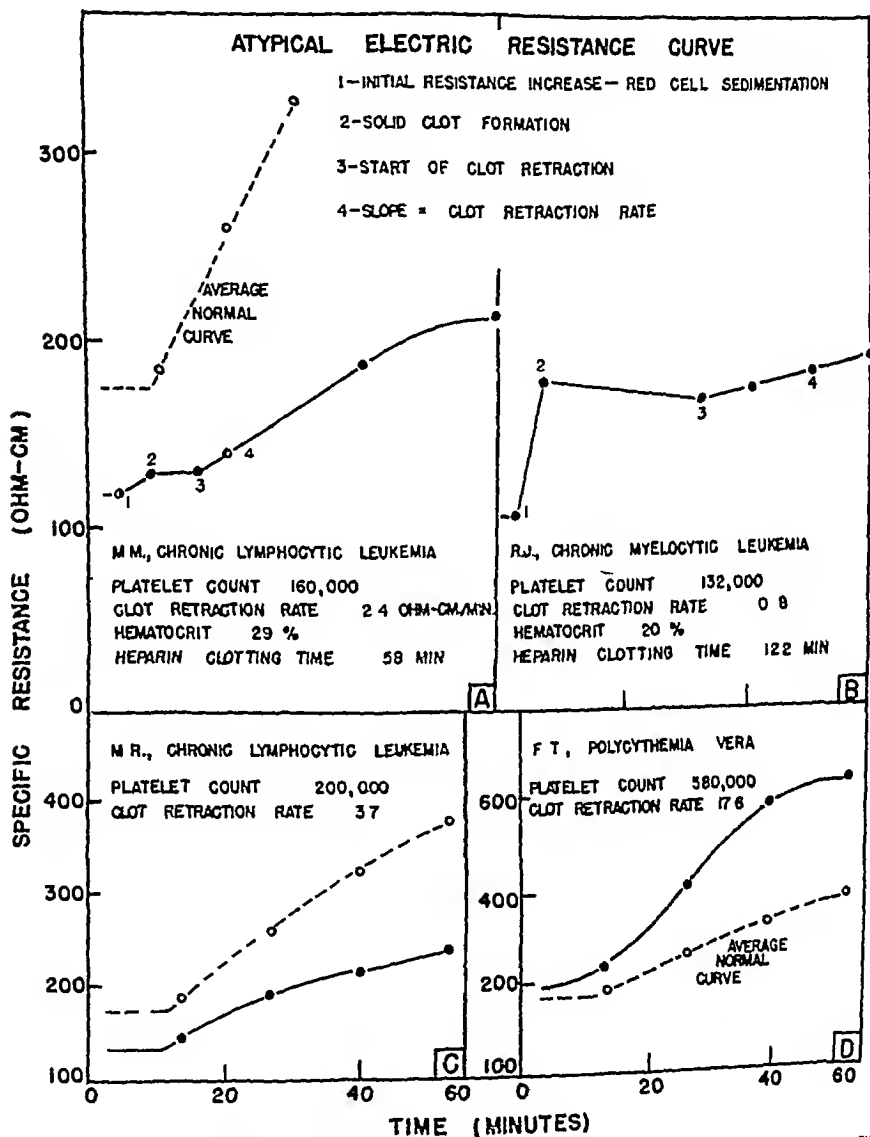


Fig 3—A and B Examples and explanation of the atypical electric resistance curve. C and D Examples of typical electric resistance curves which gave decreased and increased clot retraction rates respectively.

**Heparin Clotting Time**—The results of this test are shown in Fig 4. Thirty-three normal subjects showed a normal distribution of values. Eleven of twenty-one leucemic patients showed a definite prolongation of varying degree up to 155 min, as compared with the upper limit of normal of 35 minutes.

In polycythemia vera, twenty four patients had normal values three values were rapid and eight prolonged. The effect of an elevated hematocrit upon these results merits special consideration. Since the standard amount of heparin is added to a constant volume of blood samples with higher hematocrits will have a correspondingly higher heparin concentration in the plasma. As red cells do not appear to play any active part in the clotting process the higher heparin

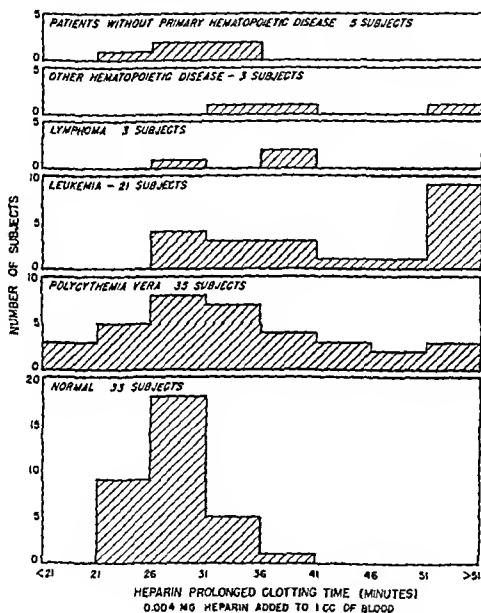


FIG. 4.—Distribution of results of the heparin clotting time (or heparin prolonged clotting time) for the various groups of subjects

concentration in the plasma could conceivably increase the heparin clotting time. This was found to be true for one patient who had hematocrits of 71, 63, 58 and 49 per cent at the times of study. Correction of the heparin clotting times for hematocrit caused a shift of five patients in the normal range to the rapid range.

In the other groups patients with lymphomas and other hematologic disease tended to show prolonged values while all patients without any primary hematopoietic disease were in the normal range.

**Clotting Time (Ice White).—**As shown in Fig. 5, all the patients showed normal values for this test.

TABLE II COAGULATION AND OTHER DATA FOR LEUCEMIC PATIENTS

SUBJECT	SEX	TYPE OF LEUCEMIA	DATE 1 Y. AMINED (MO. YR.)	HEPARIN CLOTTING TIME (MIN.)	CLINICAL EVIDENCE OF BLEEDING	CLOT RETRACTION RATE (OHM CM PER MIN.)	CLOTTING TIME (ICE WHITE) (MIN.)	PLATELET COUNT ( $\times 10^9$ )	BLEEDING TIME (MIN.)	RED CELL COUNT ( $\times 10^6$ )	HUMATO CRIT (%)	WHITE CELL COUNT	STATUS IN 1 Y.
<i>Group 1 Heparin Clotting Time Below 40 Min</i>													
G S	F	CML	6 48	26	0*	10.2	8	200	8	4.70	44	17,300	L
E W	F	CML	4 48	27	0	8.6	6	520	2 1/2	4.00	35	44,500	D
W M	M	CLL	4 48	28	0	-	9	290	-	5.74	56	60,000	L
V C	F	CML	10 48	29	0	12.3	6 1/2	360	-	3.62	40	69,000	L
R S	F	CML	10 48	32	0	7.7	8 1/2	280	-	4.28	43	60,000	L
G H	M	CLL	12 48	34	0	9.0	7 1/2	220	-	4.60	45	27,800	L
A J	M	CLL	10 48	35	0	5.4	9	200	-	4.84	47	54,800	L
G H	F	CLL	9 48	38	0	7.0	8 1/2	210	-	4.16	39	25,300	L
W H	M	CLL	9 48	38	0	7.5	8 1/2	200	-	3.80	41	34,000	L
I I	F	CML	10 48	40	0	9.1	6 1/2	360	-	3.50	36	125,500	L
<i>Group 2 Heparin Clotting Time Over 40 Min</i>													
L W	M	ALL	4 48	44	+	3.6	6	300	2	3.12	34	20,500	D
L B	F	CML	10 48	47	++	1.9†	6	230	3 1/2	2.08	20	54,000	D
H W	M	CLL	4 48	53	0	5.1	8	120	5	4.46	43	21,900	L
T P	M	CLL	6 48	58	0	6.8	6	300	-	4.10	41	16,800	L
M M	M	CLL	9 48	58	0	2.4†	8	160	3	2.90	29	10,300	L
P B	M	CLL	5 48	62	+	6	5 1/2	180	-	3.80	32	37,000	D
C S	F	CLL	6 48	118	++	4.7	8	160	-	3.74	38	29,750	L
R R	F	CLL	9 48	122	++	8†	9 1/2	130	20	2.10	20	7,950	L
I C	M	CLL	4 48	130	++	1.6	7	180	5	3.40	36	8,100	D
R I	M	CML	4 48	154	+++	1.5†	4	110	11	1.73	20	90,000	D
I H	M	CLL	4 48	155	++	1.1	7	160	8	2.80	27	30,000	L

ALL Acute leucemia

CML Chronic myelocytic leucemia

CLL Chronic lymphocytic leucemia

+ Slight bleeding, ++ moderate bleeding, +++ marked bleeding

D Dead 1 living

\*Vital bleeding subsequently discovered to be caused by uterine carcinoma  
†Atypical electric resistance curve

TABLE III. COAGULATION AND HEMATOLOGIC DATA FOR POLYCYTHEMIA VERA PATIENTS WITH INCREASED CLOT RETRACTION RATES

PATIENT	SEX	DATE (MO. YR.)	CLOT RE- TRACTION RATE (OHV CM PER MIN)	PLATELET COUNT (CU MM)	HEPARIN CLOTTING TIME (MIN)	CLOT TING TIME (MIN)	RED CELL COUNT ( $\times 10^6$ ) (CU MM)	HEMATO- CRIT (%)	WHITE CELL COUNT (CU MM)
A F	F	10 47	10.2	080 000	-	9	5.72	45	18 800
H M	M	9 48	10.3	200 000	29	9	7.34	66	16 600
L G	M	4 48	10.5	600 000	26	7	8.16	69	14 200
A P	M	10 47	11.1	470 000	-	8	7.70	58	21 800
H K	M	4 48	12.3	350 000	31	6	5.54	51.5	13 500
T F	M	0 48	13.2	400 000	25	6	5.12	52	12 500
F W	F	10 48	13.9	480 000	25	11	6.84	60	39,000
F B	M	11 48	13.9	580 000	20	8	8.30	61	33 000
G T	M	6 48	15.0	376 000	17	5	6.16	65	12 000
F K	F	10 48	15.1	490 000	43	10	7.45	62.5	10 300
C R	M	10 48	15.7	540 000	24	9	6.88	64	35 500
F T	M	11 48	17.6	580 000	24	8	6.42	56	13 750

In the clot retraction studies inspection of the clots frequently showed little or no evidence of clot retraction, while, at the same time the electric resistance curves indicated abnormally rapid retraction. When the electrodes were removed after twenty-four hours a small, firm clot was often found well retracted between the electrodes. This was surrounded by red cells suspended in serum which composed most of the volume of the sample. This tended to occur chiefly in patients with both elevated hematocrits and platelet counts. These patients also gave poor end points of clotting time and some times a solid clot either never formed or rapidly dissolved.

Four patients in hematologic remission had normal clot retraction rates. Platelet counts were normal in three patients slightly depressed below 200,000 in one. Heparin clotting time was normal in two and prolonged in two. Of the two patients in the leucemic phase of polycythemia one had a depressed platelet count and low clot retraction rate, while the other had normal values for these tests and for the heparin clotting time.

*Other Diseases* Because the number of patients in this category is small and the types of diseases varied only some general observations will be made. Patients with lymphomas and other diseases related to the hematopoietic system generally showed some bone marrow depression as evidenced by anemia and slight thrombocytopenia. None had any sign of hemorrhage. These patients tended to have prolonged heparin clotting times and lowered clot retraction rates. Patients without hematologic disease and without evidence of bone marrow depression had normal platelet counts, clot retraction rates and heparin clotting times. The two patients with polycythemia secondary to congenital cardiac abnormalities had normal values for these tests.

#### DISCUSSION

The results of this study have revealed certain new findings relative to blood coagulation in leukemia and polycythemia vera.

One half of the patients with either chronic myelocytic or chronic lymphocytic leukemia showed a blood coagulation defect characterized by slight

thrombocytopenia (100,000 to 200,000), diminished clot retraction rate, and prolonged heparin clotting time. This defect was closely related to the presence of clinical evidence of bleeding. The severity of the bleeding appeared to be especially related to the degree of heparin clotting time prolongation and, to a lesser extent, to the degree of depression of the clot retraction rate. The platelet count, bleeding time, and tourniquet test showed less relation to the clinical picture of hemorrhage, while the Lee-White clotting time was entirely unrelated. It should be emphasized that these patients did not have the marked purpura, hemorrhages, or thrombocytopenia (below 80,000) frequently associated with acute leukemia or chronic leukemia in the terminal stage. Study of a few patients in these categories indicates the above mentioned clotting changes to be present in even greater degree.

Leukemic patients without any evidence of bleeding showed normal values for the coagulation tests and platelet counts above 200,000. These patients, in comparison with the foregoing group, appeared to be in better condition, both hematologically and clinically. This is further indicated by the fact that one of ten patients in this group had died as of January, 1949, while among those patients showing the coagulation defect, five of eleven had died. This is compatible with the observations of Minot and Buckman<sup>17</sup> and others that the level of platelets appears related to the general status of the leukemic patient, with a progressive thrombocytopenia indicative of a poor prognosis.

In polycythemia vera, it was noted that the blood of patients with elevated hematocrits and platelet counts formed fragile, rapidly dissolving clots which appeared to have poor retraction. Electric resistance measurements and careful inspection, however, often indicated the presence of a small, well retracted clot surrounded by a red cell suspension in serum. With a relatively small amount of serum present, a widely latticed fibrin network clot probably is formed. Under the influence of the large number of platelets, clot retraction occurs at a rapid rate but fewer red cells are caught in the network so that the retracted clot becomes very small, leaving most of the red cells suspended in serum at the periphery to give the appearance of poor clot retraction, as recorded in the literature. This defective clot formation contributes to the bleeding symptomatology in polycythemia. Hence, the thrombocytosis, combined with the high hematocrit and increased blood viscosity, is an important factor in both the hemorrhagic and thrombotic complications. The comparable incidence of history of hemorrhage or thrombosis, increased platelet count, and increased clot retraction rate in 33 per cent of these patients merits particular note. It emphasizes the importance of the platelets and the urgent necessity of reducing an elevated platelet count in the treatment of polycythemia vera to avoid serious complication from thrombosis or hemorrhage. The depressant effect of  $P^{32}$  and other radioactive isotopes upon platelet formation enhances their value as therapeutic agents in polycythemia vera.

The mechanism of the prolongation of the heparin clotting time, which is indicative of an increased clotting sensitivity of the blood to added heparin,

cannot be fully explained. It involves incompletely understood effects of heparin upon the clotting mechanism and the factors in the blood which can either inactivate heparin by combining with it or which directly antagonize its anticoagulant properties. On the basis of the present study and a critical review<sup>18</sup> of the literature it appears that the platelets play a very important role in determination of the resultant effect of heparin upon the clotting time. This is indicated by statistical analysis of the data in the present study which revealed positive correlations between the heparin clotting time and both the clot retraction rate and platelet count. This is further seen in the occurrence of a prolonged heparin clotting time chiefly in patients with hematologic evidence of bone marrow depression, thrombocytopenia and impaired clot retraction. Studies to determine the relative effects of platelets and heparin upon the clotting mechanism are in progress.

The suggestion by Allen and associates<sup>19</sup> that in bleeding associated with thrombocytopenic purpura an increased amount of heparin or similar substance may be in the blood appears unlikely. In two leucemic patients with marked hemorrhages the toluidine blue titration test was negative for free or increased heparin in the blood. One of these patients had a markedly prolonged heparin clotting time of 154 minutes. It is improbable that even a minimal blood heparin increase which is not great enough to be detected by any present method could approach in importance the role of the thrombocytopenia as a causative factor in the hemorrhagic symptoms of these diseases.

The results of this study indicate the relative value of the various coagulation tests or indices. The platelet count, bleeding time and tourniquet test for capillary fragility are liable to both subjective and inherent errors and, at best, give only approximate and qualitative information. The prothrombin time shows significant alterations in a limited type of hemorrhagic dyscrasia as seen in severe liver damage. The Lee White clotting time reveals clotting abnormalities in only a small fraction of conditions of which hemophilia is the most prominent. The limited value of this widely used test can not be overemphasized.

The clot retraction rate obtained by electric resistance measurements provides both an objective and quantitative measure of clot retraction, the importance of which has suffered through a lack of adequate means for its measurement. It has been shown that the electric resistance method eliminates the effect of certain variable factors such as hematocrit and erythrocyte sedimentation rate which often prevent reliable evaluation of clot retraction by inspection. There is a high correlation between platelet count and clot retraction rate. Its clinical use is limited by the equipment required but a portable instrument is in the process of development.

The heparin clotting time appears to give an extremely sensitive and quantitative coagulation measurement in close agreement with the clinical findings. The value of this test in the detection of accelerated clotting in thrombotic conditions has long been realized<sup>20</sup> but it has been of little clinical value because of inadequate standardization and simplification. The

method presented in this report has been devised to eliminate these difficulties. The test can readily be adapted to routine clinical use for the detection of either a rapid or slow clotting process. In addition to its diagnostic value, it can serve as a valuable therapeutic guide.

#### SUMMARY

1 Two methods for the measurement of blood coagulation are presented: the heparin clotting time and the clot retraction rate. The heparin clotting time measures the clotting sensitivity of the blood to added heparin. The clot retraction rate is a quantitative measure of clot retraction obtained by electric resistance measurements. Both tests were well standardized on a large number of normal subjects. Clearly defined upper and lower limits of normal were established.

2 In chronic leucemia, both myelocytic and lymphocytic, one half of the patients showed a previously unreported coagulation defect characterized by a prolongation of the heparin clotting time, decreased clot retraction rate, and a slight thrombocytopenia between 110,000 and 180,000. The severity of this defect was closely related to the degree of hemorrhagic symptoms. No free or neutralizable heparin was detected by a toluidine blue titration test in the blood of two patients with marked hemorrhagic symptoms and the foregoing clotting defect. Leukemic patients without this clotting defect and thus with normal values for the coagulation tests showed no evidence of bleeding. These patients were in better condition, both hematologically and clinically, and had a better prognosis than patients with the coagulation defect.

3 Of the untreated patients with polycythemia vera and elevated hematocrits, 33 per cent showed increased clot retraction rates and platelet counts elevated above 400,000. A similar percentage of these patients had histories of either thrombosis or hemorrhage. An explanation is given for the simultaneous presence of an increased clot retraction rate, external appearance of poor clot retraction, and the formation of fragile, readily dissolving clots in many of the patients. These studies indicate that the elevation in platelet count is the most important single factor in the occurrence of both hemorrhage and thrombosis in polycythemia. Therapy should aim toward the rapid reduction of an increased number of platelets.

4 The results of this study provide indications of the relative clinical value of the various coagulation tests. The inadequacies and limitations of the platelet count, bleeding time, tourniquet test, and clot retraction estimated by inspection are shown. The extremely limited value of the widely used Lee-White clotting time cannot be overemphasized. The clot retraction rate provides a useful, quantitative measure of clot retraction but it requires special equipment for its performance. The heparin clotting time appears to be the most valuable single test or index of coagulation. Readily adaptable to both routine clinical and experimental use, it enables the detection of either abnormally increased or decreased clotting ability.

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## STUDIES ON THROMBOCYTOPEN

### I A RELIABLE TEST FOR THIS PRINCIPLE IN ORGAN HOMOGENATES AND IN URINE

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**I**N 1933 Tomlin and Puddu<sup>20</sup> discovered that an extract prepared from the spleen of a patient with idiopathic thrombocytopenic purpura contained an agent which injured the megakaryocytes in bone marrow cultures. The same principle was then found to be present not only in normal spleens, but in other normal organs as well (liver, lung, heart, lymph nodes, and omentum), although in apparently lesser concentrations. When a protein-free aqueous extract from a normal spleen was injected intravenously into rabbits, a considerable reduction of the platelets in the circulating blood became noticeable. Blood of the splenic vein contained more of this agent in comparison with blood of the splenic artery. Based on these findings, the hypothesis was advanced that thrombocytopenic purpura may be caused by an increased production of a platelet-reducing substance in the spleen. Splenectomy therefore should abolish this factor.

Troland and Lee<sup>30, 31</sup> in 1938 demonstrated that acetone extracts of three spleens of patients with Weillhof's disease decreased the platelet count sharply when injected into rabbits. They called the principle responsible for these changes "thrombocytopen." However, these workers were not able to recover thrombocytopen from normal organs (including the spleen), nor from spleens of patients with hemolytic anemia or Banti's syndrome.

Subsequently many investigators have studied this problem. Although some could confirm these findings,<sup>6, 13, 16, 17, 18, 22, 25</sup> others did not observe any platelet reduction<sup>11, 1, 19, 28</sup> or found inconsistent results.<sup>4, 10, 1\*, 3\*, 3\*</sup> The variable responses were often considered to be caused by the mode of preparing the extracts. Most investigators used acetone, although Hobson and Witts<sup>10</sup> found a suspension of the tissue particles in Ringer solution more effective. Interpretation of the results was also difficult because of the spontaneous fluctuations of the platelet counts in the test animals. Most workers used rabbits but rats<sup>4, 32, 3\*</sup> and dogs<sup>7</sup> also were employed.

Our approach to this problem was directed by the following considerations. Assuming that a thrombocytopen is manufactured by the spleen in thrombocytopenic purpura, it seems very likely that the liver—being the first organ to receive the blood coming from the spleen—might inactivate this principle. This assumption becomes even more probable when Moolten's hypothesis,<sup>13, 14</sup> that thrombocytopen may be a steroid, is taken into account. The effect of thrombocytopen may then depend not only on its concentration in the extract, but also

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on the modifying activity of the liver of the test animal. Consequently, a more reliable test for thrombocytopen may become available by injection of organ extracts into animals with liver cell damage.

These considerations seem to be borne out by our experimental studies. When organ homogenates were injected intraperitoneally into rats in which a high degree of liver cell damage was produced by carbon tetrachloride, a marked reduction of the platelet counts was consistently demonstrable. These results were not only obtained with preparations from normal and pathologic human spleens but also with material from other normal animal organs. A thrombocytopen\* was found also in urine from which it could be extracted with ether.

#### MATERIAL AND METHODS

All studies were performed on albino rats weighing between 150 and 300 grams. Male rats were used since males are more susceptible to intoxication with carbon tetrachloride than are females.<sup>8</sup> The animals were kept in separate metal cages and maintained on Purina dog chow and water. Each rat was used for one experiment only.

Blood was obtained by heart puncture under ether anesthesia, with tuberculin syringes and gauge 23 needles, which had previously been rinsed with a 3.8 per cent sodium citrate solution. About 0.2 cc of blood was transferred into a small paraffin dish containing a few crystals of an ammonium and potassium oxalate mixture.<sup>9</sup> The blood was stirred gently with a paraffin stick to achieve equal distribution of the formed elements. Films were prepared from the last remaining drop of blood in the syringe.

In each instance the red and white cell counts were determined besides the enumeration of the platelets. For the latter the method of Rees and Ecker<sup>10</sup> was used. The hemocytometers were kept for a period of twenty minutes in a wet chamber to assure proper settling of the thrombocytes without loss of fluid. All platelet counts were performed simultaneously by two workers and the results averaged.

Normal values established with these procedures are: RBC 7.36 million  $\pm$  1.3; WBC 16,300  $\pm$  6,000; platelets 500,000 to 900,000 per cubic millimeter. Rarely an animal was encountered showing a platelet count below 500,000. Such rats were considered unsuitable for the experiments. Ether anesthesia did not influence the results.<sup>5</sup>

Evaluation of the platelet count must take into account that the number of thrombocytes in the same animal may vary greatly, the range lying between 500,000 and 900,000. The same figures represent the normal values found in the rat population as a whole. Normal animals used in this study never had a platelet level below half a million. Consequently, a decrease was considered significant only when the thrombocytes were reduced below this value.

*Production of Liver Cell Damage*—Carbon tetrachloride (0.5 cc per kilogram) was injected intraperitoneally on alternate days according to the method of Brauer and Root.<sup>11</sup> This procedure assures severe liver cell damage with only minor injury to the kidneys. Two injections were found to be sufficient to secure adequate responses to thrombocytopen. If desired the liver cell damage may be maintained by repeated administrations of carbon tetrachloride. Blood films from such animals very frequently showed many target cells. Target cells are known to occur also in human parenchymatous liver disease.<sup>12</sup>

*Preparation of Organ Suspensions*—After removal from the organism the organs were immediately wrapped in a clean cloth and placed on dry ice in a deep freeze unit. For preparation of the saline suspension the organs were defrosted, weighed and cut into small pieces. These were mixed with double the volume of physiologic saline solution in a Waring Blendor gradually set to maximal speed for a period of ten minutes. The saline suspension

\*For the sake of convenience and because of its use in the literature the term *thrombocytopen* will be used throughout to denote a chemically unknown factor or factors which depress the platelet count in the test animal.

TABLE I EFFECT OF DURACILLIN AND OF CARBON TETRACHLORIDE ON THE PLATELET COUNT OF RATS

TABLE I. EFFECT OF DURAQUIN AND C.																	
DRUG TESTED	NUMBER OF INJECTIONS	NUMBER OF RATS USED	AVERAGE COUNTS												AVERAGE % DECREASE OF PLATELETS		
			RBC (MILLIONS)				WBC (THOUSANDS)				PLATELETS (THOUSANDS)						
			BEFORE INJECT	AFTER FIRST INJECT	AFTER LAST INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER LAST INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER LAST INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER LAST INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER LAST INJECT
			None	None	None	None	None	None	None	None	None	None	None	None	None	None	None
Duraquin	1	4	7.4	7.1	-	13.2	11.8	-	655	776	-	-	-	-	-	14	
	2	4	7.4	7.1	6.9	20.2	16.5	16.1	668	792	830	830	830	830	830	10	
Carbon tetrachloride	(On consecutive days)																
	On alternate days	7	7.6	-	7.6	16.4	-	10.9	755	-	652	652	652	652	652	16	
	2	22	7.2	-	7.2	13.2	-	11.8	713	-	643	643	643	643	643		
	3	7	7.3	-	7.5	14.7	-	15.2	786	-	662	662	662	662	662		

was then filtered first through two layers and then through four folds of gauze. After this the suspension was put back into the freezing unit and defrosted completely for each particular use.

*Procedure for the Determination of the Effect of Organ Suspensions in the Rats With Liver Cell Damage*—Liver cell damage was first produced by two applications of carbon tetrachloride. Two days following the last injection the red cell, white cell and platelet counts were determined. Then each animal was injected intraperitoneally with 10 cc of the organ suspension. Furthermore carbon tetrachloride was given to maintain the liver injury to prevent the development of any infection, 0.1 cc of procaine penicillin (Duracillin Lilly) was also injected subcutaneously. The next day the counts were repeated. If the effect was minimal the animals were reinjected with the homogenate and also with Duracillin. Counts were determined again the following day.

For the evaluation of each suspension a group of at least four rats was used. Significant changes of the red and white cell counts were hardly ever noted. These findings preclude the interpretation that the observed reduction in the number of platelets was caused by hemodilution. Occasionally the organ suspension was quite toxic and the animals died within a few hours after the injections.

The platelet counts of each group of rats before and after the injections were averaged and the decrease was expressed in per cent of the average platelet count. By this procedure the individual variation in the reaction of the animals toward the active principle contained in the material were taken into account.

## RESULTS

*1 Effect of Penicillin and of Carbon Tetrachloride on the Platelet Count*—Procaine penicillin (Duracillin 0.1 cc) which was used prophylactically throughout the experiments does not produce thrombocytopenia (Table I). The apparent increase of the platelet level lies within the range of daily fluctuations.

Carbon tetrachloride was tested in thirty six animals. The slight decline in the average platelet count (Table I) is insignificant since no animal showed a decrease below the range of normal even if four injections of the liver poison were given. Both drugs did not influence the red and white cell counts significantly.

*2 Comparison of the Effect of Injected Organ Suspensions on the Platelet Counts of Normal Rats and Rats With Liver Cell Damage*—Seven organ suspensions were studied. Ten cubic centimeters of the homogenized organs were injected in each instance. Table II summarizes the values obtained. Each value represents the average platelet count of at least four animals.

The organ suspensions when injected into normal rats left the platelet level practically unchanged with the exception of the material prepared from normal dog kidneys. In this experiment (Experiment 7) the first injection brought the thrombocyte count definitely out of the normal range and a second injection produced a further considerable decrease.

In the rats with liver cell damage the first injection consistently lowered the platelet counts significantly. Following a second injection the decrease was still more pronounced with the exception of Experiment 4 in which the platelet level returned to normal. This was the only observation of this kind encountered in this study. Ordinarily a second injection of potent material when given to rats with liver cell damage, either maintains or even further decreases the thrombocytes in the circulation.

TABLE II COMPARISON OF THE EFFECT OF ORGAN HOMOGENATES ON THE PLATELET COUNT OF NORMAL RATS AND OF RATS WITH LIVER CELL DAMAGE\*

EX PERI MENT	ORGAN	LIVER CELL DAMAGED RATS						NORMAL RATS				
		AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS			AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS	
		BE FORE IN JECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT		BE FORE IN JECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT
1	Human spleen (Idiopathic thrombocytopenic purpura)	660	376	—	43	—		749	689	—	8	—
2	Human spleen (Hodgkin's dis ease with symp tomatic thrombo cytopenic pur pura)	670	247	—	63	—		590	622	666	None	None
3	Human spleen (Lymphosarcoma with symptoma tic thrombo cytopenic pur pura)	630	370	295	41	53		759	674	790	11	None
4	Human spleen (Splenic vein thrombosis)	612	341	527	44	14		714	650	610	9	15
5	Human spleen (Paroxysmal nocturnal hemo globinuria)	600	236	—	61	—		718	—	728	—	None
6	Dog spleen (Normal)	615	354	247	42	60		614	676	620	None	None
7	Dog kidney (Normal)	599	212	—	65	—		704	476	186	32	74

\*Each value represents the average of at least four animals

The findings seem to indicate that liver cell damage permits a principle in the organ suspensions to exert its effect on the platelet level. Apparently the normal liver renders this thrombocytopenic agent ineffective. Contrary to our expectations, however, the suspensions prepared from the spleens of patients with normal platelet counts (Experiments 4 and 5) seemed to be as potent as those obtained from the spleens of patients with severe thrombocytopenic purpura (Experiments 1, 2, and 3). Furthermore, thrombocytopen was equally demonstrable in the normal dog spleen and was apparently present in the highest concentration in normal dog kidneys, since the latter material produced thrombocytopenia even in normal rats.

At this point in our investigation it was decided to use only rats with liver cell damage as test animals in further experiments. It also was felt that to secure the evidence so far obtained, more organ suspensions of various types should be tested for the presence of the thrombocytopenic principle.

3 *Effect of Injected Homogenized Normal and Pathologic Spleens on the Platelet Count of Rats With Liver Cell Damage*—Table III summarizes the results

TABLE III EFFECT OF HEMOGENIZED NORMAL AND PATHOLOGIC HUMAN SPLEENS ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE

EXPERIMENT	DIAGNOSIS	AVERAGE COUNTS												AVERAGE % DECREASE OF PLATELETS	
		RBC (MILLIONS)			WBC (THOUSANDS)			PLATELETS (THOUSANDS)							
		BEFORE INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	BEFORE INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT
1	Traumatic rupture (normal spleen)	46	73		184	186	-	382	283		51	-			
2	Hyperinsulinism (normal spleen)	70	74		192	192	-	304	197		64	-			
3	Idiopathic thrombocytopenic purpura	69	57	-	119	149	-	660	370	-	43	-			
4	Idiopathic thrombocytopenic purpura	74	72	73	180	196	161	546	350	207	36	62			
5	Hodgkin's disease (symptomatic thrombocytopenic purpura)	71	69	-	121	107	-	670	247	-	63	-			
6	Lymphosarcoma (symptomatic thrombocytopenic purpura)	78	82	74	139	141	174	630	370	293	41	53			
7	Banti's syndrome (symptomatic thrombocytopenic purpura)	78	75	-	207	101	-	697	344	-	51	-			
8	Liver cirrhosis (symptomatic thrombocytopenic purpura)	74	76	-	176	180	-	735	468	381	36	48			
9	Thrombotic thrombocytopenic purpura	74	63	-	213	127	-	633	351	-	45	-			
10	Splenic vein thrombosis	77	77	73	175	197	226	612	341	527	44	14			
11	Hodgkin's disease	74	73	-	166	169	-	633	366	-	44	-			
12	Gaucher's disease	75	74	-	178	176	-	637	314	-	52	-			
13	Acquired spherocytic hemolytic anemia	78	75	74	149	161	172	648	322	380	20	40			
14	Acquired spherocytic hemolytic anemia	7	72	73	178	17	185	616	414	334	33	54			
15	Congenital spherocytic hemolytic anemia	75	74	74	159	152	172	617	382	380	38	38			
16	Paroxysmal nocturnal hemoglobinuria	74	73	-	171	171	-	600	236	-	61	-			

\*Each value represents the average of at least four animals

All spleens were obtained during life with the exception of the case of thrombotic thrombocytopenic purpura<sup>26</sup>. In this instance the organ was received one hour after the death of the patient. The few values previously mentioned in Table II are included in this summary for a more convenient survey of the whole material studied. Regardless of the weight of the spleen and the presence of pathologic tissues (Hodgkin's granuloma, lymphosarcoma, Gaucher's disease, etc.), 10 cc of the homogenized suspension were always injected. As can be seen from Table III, all suspensions lowered the platelet level considerably. Usually a single injection produced a reduction of more than 40 per cent. If the decrease was less than 40 per cent of the preinjection level, a second injection was given, which in most instances caused a further decline of the thrombocyte count. The spleens of the patients with spherocytic hemolytic anemias seemed to contain comparatively less of the thrombocytopenic agent, although it still was readily demonstrable. Suspensions from the normal spleens caused approximately the same reduction of the level of thrombocytes as could be achieved with some suspensions prepared from pathologic spleens. No correlations were demonstrable between platelet reduction in the patient whose spleen was used for the injection and the degree of platelet decline in the injected animals. Although various pathologic spleens were used in this series of experiments, none of the homogenates produced any significant changes in the red or white cell counts.

*1 Effect of Normal Organ Homogenates on the Platelet Count of Rats With Liver Cell Damage*—The results are compiled in Table IV. The values show convincingly that the thrombocytopenic agent is not only present in dog and beef spleen, but may also be found in homogenates prepared from lungs, heart, and kidneys of a healthy dog. The suspension of lung tissue caused the severest depression of the platelet level, namely a reduction of 75 per cent of the original count.

TABLE IV EFFECT OF SUSPENSIONS OF NORMAL ANIMAL ORGANS ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

ORGAN SUSPENSION PREPARED FROM	AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS	
	BEFORE INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT
Dog spleen 1	615	354	247	42	60
2	616	247	198	60	68
3	593	377	286	36	52
Dog kidney 1	599	212	—	65	—
2	595	231	210	61	64
Dog heart	660	286	—	57	—
Dog lung	613	156	—	75	—
Beef spleen	623	411	338	34	46

\*Each value represents the average of at least four animals.

*5 The Dose-Response Relationship Between Injected Organ Suspensions and Reduction of the Platelet Count*—For the evaluation of our findings, the problem is of obvious importance whether some correlation exists between the amount of homogenate injected and the demonstrable fall of the platelet level.



Doses of 10, 8, 6, 4, 2, and 1 cc of the same saline suspension prepared from a normal human spleen were injected into groups of at least four rats with liver cell damage. The results are compiled in Table V.

TABLE V DOSE RESPONSE RELATIONSHIP BETWEEN AMOUNT OF HOMOGENATE INJECTED AND DECREASE OF PLATELET COUNT

AMOUNT OF SUSPENSION (NORMAL HUMAN SPLEEN) INJECTED (CC)	RATS USED	AVERAGE % DECREASE OF PLATELETS
10	4	64 ± 1 <sub>2</sub>
8	4	61 ± 2 <sub>2</sub>
6	5	41 ± 13
4	4	35 ± 4
2	4	43 ± 11
1	4	24 ± 24

As can be seen from the table the two largest doses (10 and 8 cc) produced the greatest decline of the platelet count 64 and 61 per cent respectively. However, the response to 2 cc and 6 cc of the suspension was approximately the same (35 to 43 per cent). The smallest dose of 1 cc elicited also the slightest effect, i.e. 24 per cent.

These results show that the dose response relationship is not a linear one but that within certain ranges larger amounts of the suspension produce greater responses. Since these studies were performed with crude homogenates, it was not considered advisable to pursue the problem further. With the isolation and purification of thrombocytopen, the rat with liver cell damage promises to become a reliable test animal for the quantitative bioassay of the platelet reducing agent.

*6 Effect of Hemoglobin Solutions and of Thromboplastin on the Platelet Count of Rats With Liver Cell Damage*—It was noticed that many animals developed hemoglobinuria following the injection of the organ homogenates. In the course of the freezing and defrosting processes employed in the preparation of the suspensions the blood contained in the organs becomes hemolyzed. Thus a relatively large quantity of free hemoglobin and erythrocyte stroma was injected into the rats with liver cell damage together with the homogenized tissues. The question arose whether the thrombocytopenia in the animals might not be caused by this hemoglobinemia. In cases of severe hemoglobinemia in human beings regardless of the etiology thrombocytopenia is frequently observed.<sup>24</sup>

Therefore 10 cc of a 3 per cent suspension of washed dog erythrocytes hemolyzed by addition of distilled water were injected into the animals. As can be seen from Table VI (Experiments 1 and 2) this procedure was followed by an insignificant drop of the thrombocyte level.

The very conspicuous reduction of the platelet level with the lung suspension (Table IV) led us to consider the possibility that thromboplastin may be identical with the thrombocytopenic agent. Thromboplastin is another ubiquitous tissue factor known to be present in particularly high concentration in the lungs. Consequently Thromboplastin Maltine a rabbit lung preparation as well as rabbit brain thromboplastin made according to Quick's method<sup>25</sup>

TABLE VI EFFECT OF HEMOGLOBIN SOLUTIONS AND OF THROMBOPLASTIN ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

EXPERIMENT	PREPARATION INJECTED	AVERAGE PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS	
		BEFORE INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT
1	Laked dog blood	636	550	-	13	-
2	Laked dog blood	630	-	545	-	15
3	(a) Suspension of dried rabbit lung	583	178	-	69	-
	(b) Milky supernatant fluid (rich in thromboplastin)	604	468	-	22	-
	(c) Granular residue (poor in thromboplastin)	517	161	-	69	-
4	(a) Suspension of dried rabbit brain	545	323	-	40	-
	(b) Milky supernatant fluid (rich in thromboplastin)	576	409	-	29	-
	(c) Granular residue (poor in thromboplastin)	539	256	-	53	-

\*Each value represents the average of at least four animals

was investigated. For each single rat experiment, 200 mg of the lung or brain powder were first suspended in saline and then injected as a whole. In another experiment the same type of organ emulsion was incubated at 56° C for fifteen minutes and then centrifuged at low speed for five minutes. The supernatant milky fluid was injected separately as was a suspension of the granular material found at the bottom of the centrifuge tube. Determination of the prothrombin times revealed that the former contained much more thromboplastin than the latter (13 and 30 seconds respectively).

The results of these experiments may be seen in Table VI (Experiments 3 and 4). Since the supernatant fluid, richer in thromboplastin, is evidently poorer in thrombocytopen, the conclusion seems justified that these two factors are not identical.

These experiments furthermore show that thrombocytopen cannot be extracted satisfactorily from organs by means of acetone since the thromboplastin preparations were made by macerating the organs under acetone and discarding the acetone extract. However, the remaining dry powder still contained a very potent thrombocytopenic agent.

7 *Effect of Normal Urine and the Urine of Patients With Thrombocytopenic Purpura on the Platelet Count of Rats With Liver Cell Damage*—Our experiments have demonstrated that thrombocytopen is inactivated by the liver. Inactivation of metabolites often occurs by conjugation with acids in the liver and subsequent excretion of the substances in the urine. When 10 cc of the concentrated morning urine of two healthy male persons were injected into rats with liver cell damage, definite reductions of the platelet counts were noticeable. The same results were obtained with urine specimens from two female patients with severe thrombocytopenic purpura.

The thrombocytopenic agent could be extracted from the urine specimens with ether. Extraction was performed by adding 3 parts of ether to 1 part of urine in a separatory funnel and shaking for ten minutes. This procedure then was repeated twice. From the combined extracts the solvent was evaporated at room temperature and the dark and sticky residue was dissolved in the appropriate amounts of carbon tetrachloride needed for maintaining the liver cell damage in the rats. By addition of saline a good emulsion could be obtained with shaking, which permitted the application of the correct doses of the various substances. It should be emphasized that no change of the platelet count was seen when carbon tetrachloride and the thrombocytopenic free urine residue were injected (Table VII).

The demonstration that the platelet reducing agent can be completely recovered from the urine with ether will probably permit quantitative estimations of the urinary output of this factor in normal and various pathologic conditions. Such studies will be reported later. So far the experiments summarized in Table VII mainly show that thrombocytopen is excreted in the urine.

When the ether extract of larger amounts of urine was injected into normal rats (extract residue of 175 cc of pooled urine suspended in saline and injected per rat) no significant reduction of the platelet level was observed. These results are in agreement with the findings of Tocantius,<sup>48</sup> who could not decrease the thrombocytes of normal rabbits with urinary extracts. The detoxifying action of the normal liver apparently is very efficient. The amount of thrombocytopen able to reduce the platelet count in normal animals remains to be determined. Such studies may better be performed when sufficient quantities of the purified material are available.

*8 Mode of Action of Thrombocytopen*—Although the reduction of thrombocytes following the injection of organ homogenates is sometimes a considerable one we have never noticed the development of true purpuric lesions in our experimental animals. This finding is not an unexpected one. Roskan<sup>21</sup> as well as Bedson showed that by intravenous injection of gelatin or agar extreme thrombocytopenia may be produced without any increased bleeding tendency. However, if the capillaries were damaged simultaneously (e.g. by antiried cell serum) purpura resulted.

From the experimental observations of Torrioli and Puddu<sup>20</sup> it is likely that thrombocytopen acts directly on the megakaryocytes by inhibiting platelet production. This view is also emphasized by Dameshek and Estren. We have postponed a systematic study of the marrow until a purified thrombocytopen is at hand. In the few instances in which the marrow was examined after injection of organ homogenates a slight increase of the more immature basophilic megakaryocytes was noted occasionally.

In a few animals the recovery phase following a single injection of an organ homogenate was studied. If after the production of thrombocytopenia all applications were stopped the platelet count returned to normal within twenty-four to seventy-two hours.

TABLE VII EFFECT OF URINE AND OF URINE EXTRACTS ON THE PLATELET COUNT OF RATS WITH LIVER CELL DAMAGE\*

TYPE OF URINE	FRESH URINE						URINE AFTER REMOVAL OF THROMBOCYTOPEN WITH ETHER						ETHER EXTRACT OF URINE					
	PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS			PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS			PLATELET COUNT (THOUSANDS)			AVERAGE % DECREASE OF PLATELETS		
	BEFORE INJECT	FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	BEFORE INJECT	FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	BEFORE INJECT	FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT	AFTER SECOND INJECT	AFTER FIRST INJECT
Normal	1	579	406	—	—	—	543	536	547	1	0	0	544	372	352	11	54	54
	2	585	432	244	58	—	523	529	460	0	11	—	545	370	240	32	56	56
Thrombocytopenic	1	593	387	272	54	—	554	580	577	0	0	0	553	285	331	48	40	40
Purpura	2	565	363	—	—	—	583	548	562	6	4	—	566	359	372	37	34	34

\*Each value represents the average of at least four animals

## DISCUSSION

The results of our experiments seem to indicate that a thrombocytopenic agent can be obtained from many different normal organs (spleen, lungs, heart, kidneys, and brain). Since consistent lowering of the platelet count is demonstrable only in animals with liver cell damage and since it also can be produced by the injection of urine, one may speculate that physiologically thrombocytopen is rendered innocuous in the liver and then is excreted in the urine. Since it can be recovered from the ether extract of the urine, this factor is probably lipid in nature. Thus the metabolic behavior of thrombocytopen resembles closely the well known pattern found with many steroid compounds.

The role which the liver plays in the inactivation of thrombocytopen may also partly explain the inconsistent or negative results of many investigators (ten out of nineteen) who worked with organ or urine extracts and injected them into normal animals. Our results permit the statement that the rat with liver cell damage is well suited for the demonstration of the thrombocytopenic agent.

Thrombocytopen has been of interest to most investigators on account of its hypothetical role in the pathogenesis of thrombocytopenic purpura. Torrioli and Puddu<sup>2</sup> have emphasized that the principle which injured the megakaryocytes in bone marrow cultures is also present in many normal organs. If thrombocytopen is identical with this factor of the Italian authors, who used a 'protein free aqueous extract' our findings confirm their observations. We have, however, not been able to demonstrate any significant differences in the degree of thrombocytopenia produced in the animals by the homogenates prepared from the various types of pathologic spleens. The results obtained with the sixteen human spleens used in this study (Table III) may be grouped in relation to the hematologic status of the splenectomized patients. With normal spleens the average platelet reduction in the animals following a single injection was 57.5 per cent; with spleens from patients with severe idiopathic or symptomatic thrombocytopenic purpura (Table III, Experiments 3 to 9) the decrease was 45 per cent; and with spleens of patients without thrombocytopenia but with other hematologic disorders (Table III, Experiments 10 to 16) the lowering of the platelets amounted to 41.7 per cent.

Since the suspensions always were prepared in the same manner and the injected dose was kept constant these results indicate that the concentration of the platelet reducing principle per gram of tissue apparently was not increased in the spleens from patients with thrombocytopenic purpura. Furthermore there was no splenomegaly in the patients with Werlhof's disease which fact rules out the possibility that the absolute amount of thrombocytopen with in these extirpated organs may have been abnormally large.

On the other hand it must be realized that crude homogenates are not too well suited for exact quantitative evaluation as may be seen from our study of the dose response relationship (Table V) in which a saline suspension prepared from a normal spleen was used. Furthermore the quantity of a biologic agent present in an organ at any given moment does not directly reflect on its

production within this organ, or on its release into the circulation. Since our demonstration of the thrombocytopenic principle in the urine did not include any quantitative estimation of the output per day, this problem requires further investigation. Nevertheless, if one takes into consideration that so many normal organs contain at least a similar concentration of thrombocytopen as can be found in normal as well as in pathologic spleens, there is no evidence available from our data to support the hypothesis that this agent may definitely be involved in the pathogenesis of thrombocytopenic purpura. However, we are not yet able to explain why some investigators, although using normal animals for testing, could demonstrate the principle more readily in pathologic spleens than in normal organs.

In this respect the work of Moolten<sup>13, 14</sup> is of particular interest. This author reported that normal and also certain pathologic spleens (e.g. in Hodgkin's disease) contain not only thrombocytopen but also another lipid factor which has the opposite effect on the platelet level. He called this antagonist of thrombocytopen, capable of increasing the thrombocyte count, "thrombocyto-sin." Both thrombocytopen and thrombocyto-sin could be obtained as relatively purified substances and formed ether-insoluble digitonides in the manner of steroids. Thrombocyto-sin also was found in high concentrations in fatty tissues and in egg yolk and was effective when given orally. Normal urine and particularly the urine of a splenectomized patient sometimes contained large amounts of the platelet-increasing agent. Moolten speculates that thrombocytopen may be concerned in balancing the thrombocyto-sin produced by the dietary factor thrombocyto-sin. Under the conditions of our experiments with rats with liver cell damage we have not yet encountered any results suggestive of an effect of thrombocyto-sin.

Ungai<sup>33, 34</sup> has isolated two substances from the spleen capable of influencing the bleeding time of guinea pigs. "Splenin A" decreases, whereas "Splenin B" definitely increases, the bleeding time. Splenin B is considered to be possibly identical with the thrombocytopen of Tioland and Lee<sup>31</sup>. However, no experimental evidence is yet available demonstrating the ability of Splenin B to reduce the platelet level. It should be of interest to test these substances in rats with liver cell damage.

So far we have assumed that the thrombocytopenic agents found in the various organ homogenates as well as in the urine are one and the same principle. There is actually no proof for such an assumption, and this approach should be considered primarily a working hypothesis. We are, however, convinced that the thrombocytopenia in the rats with liver cell damage is a specific response to a single factor or to several factors. Such an interpretation is supported by the finding that the reduction of the platelets was not accompanied by any change in red and white cell counts.

The physiologic significance of thrombocytopen is at present unknown. It should be kept in mind that the demonstration of a powerful biologic agent obtained from animal tissues does not necessarily indicate any "physiologic activity." Heparin, for instance, is certainly a very potent anticoagulant but its role in the normal coagulation process is still debatable.

The use of the rat with liver cell damage should provide a reliable tool for the study of many problems related to 'thrombocytopen'. Whatever the outcome may be, the doubts concerning the reality of such a factor or factors may now be abandoned. Their existence at least seems to be assured.

#### SUMMARY

1 Thrombocytopen is the name which has been given to a principle, present in the spleen which when injected into laboratory animals, is said to decrease their platelet count. This principle is believed to play an important role in the pathogenesis of thrombocytopenic purpura. So far nine out of nineteen investigators have confirmed the existence of this thrombocytopenic agent whereas the others obtained either inconsistent or negative results.

2 Our approach to this problem was directed by the following considerations. Assuming that thrombocytopen is manufactured predominantly by the spleen it seems very likely that the liver—being the first organ to receive blood coming from the spleen—may be involved in the inactivation of this principle. The effect of thrombocytopen may then depend not only on its concentration in the organ extracts but also on the modifying activity of the liver in the test animals. Consequently a more reliable test for thrombocytopen may become available by injection of organ suspensions into animals with liver cell damage.

3 When organ homogenates were injected into rats with a high degree of liver cell damage produced by carbon tetrachloride, a considerable drop of the platelet count was consistently produced. The same organ suspensions did not reduce the platelet count of normal animals. Neither penicillin (used for prevention of infection) nor carbon tetrachloride by themselves influenced the platelet levels. No changes in the red or the white cell counts were observed.

4 Two normal human spleens, seven spleens from patients with thrombocytopenic purpura (idiopathic and symptomatic) and seven spleens from patients without any thrombocytopenia but with other hematologic disorders were tested. All these organs contained thrombocytopen in approximately similar concentrations. The platelet reducing agent also was found in at least the same amount in normal organs (lung, heart, kidney, brain) obtained from dogs, cattle or rabbits.

5 When nine specimens of normal individuals or from patients with thrombocytopenic purpura were injected into rats with liver cell damage a significant reduction of the platelet level also occurred. The platelet reducing agent could be extracted with ether.

6 These results may indicate that physiologically thrombocytopen is rendered innocuous in the liver and then excreted in the urine. Since it can be recovered from the ether extract this factor is probably lipid in nature. Thus the metabolic behavior of the thrombocytopenic agent resembles the well known pattern of steroid compounds.

7 There is at present no evidence available from our data which would support the hypothesis that thrombocytopen is definitely involved in the pathogenesis of thrombocytopenic purpura. However, further studies are necessary.

8 The thrombocytopenic agents found in the various organ homogenates as well as in the urine have been assumed to be one and the same principle. This approach should be considered primarily a working hypothesis.

9 The use of rats with liver cell damage should provide a reliable tool for the study of many problems related to thrombocytopenia. Whatever the outcome may be, the doubts concerning the reality of such a factor or factors may now be abandoned. Their existence at least seems to be assured.

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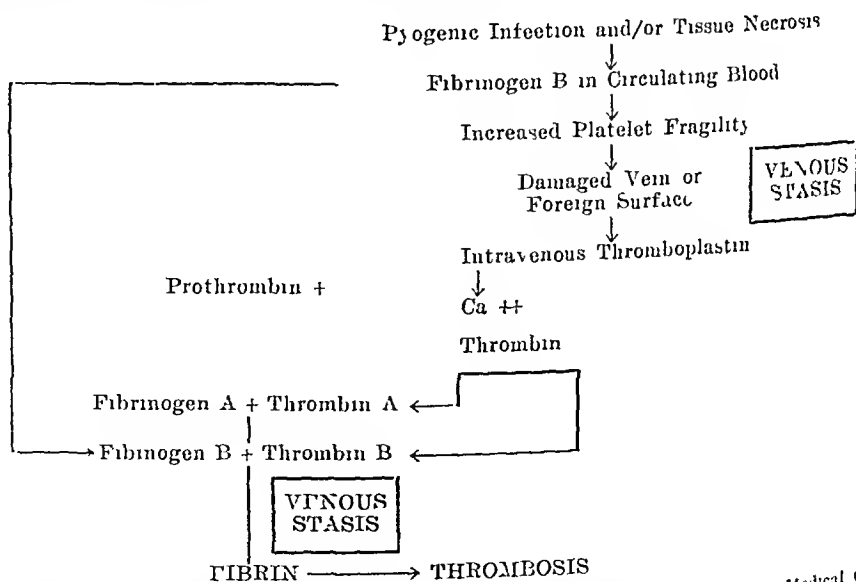
# THE FIBRINOGEN B TEST AND INTRAVASCULAR THROMBOSIS

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IN RECENT years intravascular thrombosis has assumed a prominent position on the list of postoperative complications as other more common complications have been brought under control. A laboratory method for determining the presymptomatic thrombosis would be of great value in reducing the mortality rate and serious sequelae of intravascular thrombosis. Our interest in the fibrinogen B reaction was stimulated by the possible relationship it might hold to the diagnosis of intravascular thrombosis. The fibrinogen B reaction described by Cummine and Lyons<sup>1</sup> was studied for a six-month period on the Surgical Service of Brooke General Hospital. The data set down in the report were derived from patients who had been hospitalized during this period of study.

Cummine and Lyons accepted the earlier hypothesis of Beijquist,<sup>1</sup> who stated that just prior to the formation of an intravascular thrombus a "pre thrombotic state" exists during which there is a significant reduction in venous clotting time. Although these investigators employed slightly different methods of clotting time determination, the critical zone was considered to be 3 to 4 minutes, or less.

Cummine and Lyons introduce the concept of an intermediary product in the fibrinogen-to-fibrin reaction, to which they give the name fibrinogen B. The presence of fibrinogen B in the circulating plasma is considered abnormal. They correlate its presence in the plasma with the production of intravascular thrombosis. They note that fibrinogen B appears in the plasma whenever tissue necrosis is present, especially in the case of pyogenic infection. Lyons has demonstrated in vitro that platelet fragility increases in the presence of fibrinogen B and has postulated the following possible mechanism<sup>2</sup>



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Cummie and Lyons studied 580 patients and catalogued the results of their investigation as follows<sup>1</sup>

Group 1—In these patients free fibrinogen B does not occur in the plasma and the coagulation graph is of normal type. Intravascular thrombosis does not develop in these cases.

Group 2—Free fibrinogen B may be found in the plasma either persistently or intermittently but the coagulation graph is of normal type. Providing that no previous thrombosis has occurred and is remaining as an intravascular foreign body and that venous stasis is prevented thrombosis does not develop in these cases.

Group 3—Free fibrinogen B occurs in the plasma and the coagulation times remain persistently low in the order of 3 to 4 minutes. Intravascular thrombosis appears to be inevitable in these cases either in the legs or at the lung bases.

Group 4—No free fibrinogen B is found in the plasma but the coagulation graph is consistently low. This combination of the two factors has not occurred in any of the patients studied as a primary phenomenon but has been seen on several occasions after an intravascular thrombosis has occurred. In each instance it is significant that the thrombosis recurred at the initial site and can be interpreted as a local exacerbation due to the presence of an intravascular foreign body in association with venous stasis.

We have undertaken a study for the purpose of estimating the practical value of the fibrinogen B test in a military medical establishment.

#### METHODS AND MATERIALS

*Clotting time determinations* were performed after the method outlined by Cumme and Lyons with only slight modification, i. e. the capillary tube filled with fingertip blood was broken every thirty seconds rather than every sixty seconds during the first four minutes.

*Fibrinogen B determinations* were performed as follows. The reagent was prepared by dissolving 2 Gm  $\beta$  naphthol in 100 ml of 50 per cent alcohol. If the solution turned brown, it was exposed to oxygen in order to return it to the colorless state. Blood was withdrawn from the antecubital vein in a dry sterile syringe and 4.5 ml were mixed immediately with 0.5 ml of 11 per cent solution sodium oxalate. The blood sample was taken usually midway between breakfast and lunch; however we found no evidence to suggest that the results were influenced by food intake. The specimen was centrifuged and 1 ml plasma was placed in a 13 by 100 mm tube. Five drops of the reagent were added to the plasma and the mixture was allowed to stand at room temperature for ten minutes. If a gel formed a value of from 1 to 4+ was assigned depending on the quantity of gel present. If no gel formed during that period the test was recorded as negative for fibrinogen B.

#### RESULTS

A total of 553 fibrinogen B determinations and 337 capillary clotting time determinations were made on forty-eight hospital patients and four normal subjects. The maximum length of time an individual case was followed was fifty days and the minimum one day.

The cases chosen were selected deliberately for anticipated demonstration of a wide latitude of response to the test. Table I lists by diagnosis the types of cases studied and the results of the survey in terms of "positive," "doubtful," and "negative." The extent or severity of the pathologic changes is not correlated, nor is any quantitative estimate of the amount or duration of fibrinogen B present stated. In general however the quantity of fibrinogen B present was in direct proportion to the severity of the disease.

Columns three and four show the results obtained at room temperature and at 6° C. The description of the test set down by Lyons states that room temperature is to be used. We noted that certain tests negative at room temperature would become positive if repeated at 6° C. On further investigation, it was demonstrated that as the quantity of fibrinogen B rose it could be detected earliest at 6° C and then later at room temperature. Conversely, as the plasma content of fibrinogen B fell, the test first became negative at room temperature and later at 6° C. Correctly or not, we assumed that the gel formations at room temperature and at 6° C represented the same substance, and we used this finding as a rough quantitative index.

TABLE I

DIAGNOSIS	NUMBER OF CASES	RESULTS					
		ROOM TEMPERATURE			6° C		
		P	D	N	P	D	N
Normal subjects	4	0	0	4	0	1	3
Thrombophlebitis, deep	12	3	4	5	11	0	1
Thrombophlebitis, superficial	1	0	0	1	1	0	0
Soft tissue trauma	2	1	0	1	2	0	0
Acute infections	6	0	1	5	5	0	1
Acute thermal and chemical burns	3	1	0	2	2	0	1
Chronic infection	9	0	1	8	6	3	0
Carcinoma (brain, stomach, cervix)	3	0	0	3	3	0	0
Abdominal stab wounds, contaminated	2	1	0	1	2	0	0
Uncomplicated postoperatives	5	1	0	4	2	3	0
Cirrhosis, periportal	1	0	0	1	1	0	0
Common duct obstruction	1	1	0	0	1	0	0
Myocardial infarction	1	0	0	1	1	0	0
Congestive failure	1	0	0	1	1	0	0
Late pregnancy with pyrexia (unknown origin)	1	0	0	1	1	0	0
Prolonged bed rest with hip fracture	1	0	0	1	0	1	0

P positive D doubtful N negative

Examination of the results in Table I indicates that there is no specificity of response peculiar to cases of intravascular thrombosis. Suggestive trends are poorly defined. One finding is outstanding, namely, that whenever tissue necrosis is present, the test is usually positive.

In the twelve cases of deep thrombophlebitis, only one patient demonstrated a positive test at room temperature for fibrinogen B on the day of clinical diagnosis. Subsequent positive results at room temperature were inconstant and afforded little recognized clinical value. In this series three of the twelve patients gave a clotting time of 4 minutes or less on the day of clinical diagnosis.

In the entire series of fifty-two cases, only two patients fulfilled one or the criteria of Cummine and Lyons, i.e., lowering of clotting time and the appearance of fibrinogen B in the plasma as indicative of inevitable thrombosis. Neither patient developed clinical symptoms of intravascular thrombosis.

#### DISCUSSION AND SUMMARY

Over a six-month period at Brooke General Hospital, the incidence of deep thrombophlebitis on the Surgical Service was less than 1 per cent. To conduct a survey for the purpose of extensively confirming or denying the hypothesis of

Cummie and Lyons, it would be necessary to follow daily every individual admitted to the Surgical Service that is, to make in the neighborhood of 20,000 to 25 000 tests. In the experience reported herein, 553 tests were made. In only two instances the criteria of Cummie and Lyons for inevitable thrombosis were fulfilled but neither patient developed clinical evidence for intravascular thrombosis. On the basis of experience to date the test would appear to be of no particular value in the confirmation of a diagnosis of thrombophlebitis suspected clinically. While the present small series neither confirms nor denies the concepts expressed by Cummie and Lyons it places some doubt on the precision of interpretation of low clotting times.

In light of the present good results of anticoagulant therapy in intravascular thrombosis initiated on clinical evidence alone the practicality of this test is open to question as a routine examination on all surgical patients.

The fact that the test is more sensitive at 6° C is not explained. The results are interesting but are difficult to assess.

We are in agreement with Cummie and Lyons in noting an apparent relation between the presence of tissue necrosis and the appearance of fibrinogen B in the circulating plasma. In fact this relationship is the only constant finding. We have noted also, as have Cummie and Lyons that there is a persistently negative test for fibrinogen B in thrombophlebitis after it is initially positive.

#### ADDENDUM

Dunn, Jackson and Lyons report a high percentage of positive fibrinogen B tests in congestive cardiac failure (97 per cent) recent coronary occlusion (100 per cent) thrombotic states (100 per cent) acute sepsis (100 per cent), and chronic sepsis (90 per cent).

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# DETERMINATION OF PROTHROMBIN BY THE DILUTION METHOD STABILITY AND ACTIVITY OF HUMAN AND BOVINE PROTHROMBIN-FREE PLASMA

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THE reporting of plasma prothrombin activity in terms of the time in seconds required for coagulation represents incomplete data since prothrombin activity does not vary as a linear function of the time of coagulation. However, prothrombin activity is reported properly in terms of concentration in per cent of the average concentration of pooled normal plasma. Plasma prothrombin concentration can be determined by one of two basic methods. The two stage method of Warner, Binkhous, and Smith,<sup>18</sup> an excellent research method, is too demanding on the basis of time and reagents required to be of routine clinical use. The one stage method of Quick<sup>11</sup> is generally employed because of its simplicity and rapidity of performance. The Quick method, however, determines only the prothrombin time or the speed of the reaction comprising coagulation. In order to convert the prothrombin time to prothrombin concentration it is necessary to determine the prothrombin time of known concentrations of prothrombin. By arbitrarily considering that pooled normal plasma has a prothrombin concentration of 100 per cent, then any particular dilution of the pooled plasma theoretically is equivalent to a corresponding dilution of prothrombin. In practice the prothrombin times of several known dilutions of plasma are determined and a reference curve is made, plotting the prothrombin time in seconds along the abscissa and the prothrombin concentration, in terms of per cent of normal, along the ordinate. Such a curve is then used for conversion of prothrombin time in seconds to prothrombin activity in terms of per cent concentration of prothrombin.

Because of the significant variations in the shape and slope of the reference curves using various diluents of pooled normal plasma, Rosenfield and Tuft<sup>14</sup> suggested the use of a diluent that is theoretically ideal, namely, normal plasma treated with barium sulfate to remove prothrombin. Because the resulting reference curve, using such a diluent, has a steep slope from a prothrombin concentration of 100 to 20 per cent and a flat linear slope from 15 to 1 per cent, it becomes necessary to reduce the prothrombin concentration in a given plasma to approximately 10 per cent in order to determine accurately its prothrombin concentration. This dilution is required to reduce to a minimum the error that results from variations of a few seconds when converting prothrombin time to prothrombin concentration.

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The dilution method for prothrombin determination is outlined by Rosenfield and Tuft<sup>14</sup> is accurate and reproducible and is therefore desirable. However, there have been two technical limitations of the method. The first is the rapidity with which prothrombin free plasma becomes altered on standing and the second is the unavoidable reduction of certain prothrombin accelerators<sup>7, 10</sup> by treatment of plasma with barium sulfate. Both of these alterations of barium sulfate treated plasma may result in prolongation of the prothrombin time of diluted fresh normal plasma that may be incorrectly interpreted as indicating a reduction in prothrombin concentration. The purposes of this paper are (1) to report a method whereby prothrombin free plasma can be maintained in stable form for many weeks and (2) to offer a procedure for correction of decreased content of the 'prothrombin accelerators'.

#### MATERIALS AND METHODS

**Thromboplastin**—Lyophilized acetone extracted rabbit brain (Difco) was used. Activation of the material as outlined by Difco Laboratories was effected by suspending 0.15 Gm of the lyophilized material in 4 ml of a solution of sodium chloride (0.95 Gm per 100 ml) and 0.05 ml of sodium oxalate solution (1.34 Gm per 100 ml) and incubating at 45° C for ten minutes with agitation every three minutes. After completion of incubation large particles of suspended tissue were removed by crude filtration through cotton placed loosely over the tip of a 20 ml volumetric pipette which was then lowered into the suspension and filled by gentle suction. The thromboplastin solution so obtained was stored in a deep freeze at -20° C in 0.1 ml aliquot. In this form it remains stable for many months.

**Calcium Chloride**—A solution of 0.05M calcium chloride was made by dissolving 2.77 Gm of purified anhydrous  $\text{CaCl}_2$  in 1000 ml of single distilled water.

**Anticoagulants**—One part of either of the following solutions was used to render 9 parts of whole blood incoagulable.

(a) 0.1M sodium citrate was made by dissolving 20.412 Gm of sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ ) in 1000 ml of single distilled water.

(b) 0.1M potassium oxalate was made by dissolving 18.432 Gm of crystalline potassium oxalate ( $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ ) in 1000 ml of single distilled water.

**Citrated Pooled Normal Plasma**—Citrated plasma was used for prothrombin determinations because of the greater stability of prothrombin and 'prothrombin accelerators' with this anticoagulant as compared with oxalate.<sup>3</sup> Blood samples were obtained from ten normal subjects each taken separately using 1 part of 0.1M sodium citrate for 9 parts of venous blood. The plasma obtained by centrifugation at 2000 rpm for ten minutes was pooled and designated as 'pooled fresh normal plasma (citrate)'.<sup>1</sup>

**Prothrombin Free Plasma**—Oxalated plasma was used since the prothrombin cannot be removed satisfactorily from citrated plasma.<sup>1</sup> Blood samples were obtained from ten normal subjects each sample taken separately using 1 part of 0.1M potassium oxalate for 9 parts of venous blood. Plasma was obtained by centrifugation of the oxalated blood samples at 2000 rpm for ten minutes. The samples of plasma were then pooled and 100 mg of powdered barium sulfate (Merck) were added for each 10 ml of plasma. This mixture was incubated at 37° C for ten minutes with agitation every three minutes and finally centrifuged at 3,000 rpm for thirty minutes and the supernatant removed. Such plasma is designated 'prothrombin free plasma (barium)'. It was stored at 4° C and used immediately unless otherwise stated.

Oxalated bovine plasma from bovine blood obtained by venipuncture from the living animal was rendered prothrombin free by the same process and designated 'prothrombin free plasma (bovine)'.

stable when frozen and maintained at  $-20^{\circ}\text{C}$  for relatively short periods of time, but even at this temperature of storage alteration in activity is observed after several days. The prolongation of prothrombin time is not due to quantitative changes in fibrinogen. When stored in the fluid state for limited periods, no change occurred in the fibrinogen content of prothrombin-free plasma and there was no apparent decrease in the fibrinogen "reactivity," as suggested by Loomis and Seegers,<sup>6</sup> since a "normal" thrombin time can be obtained by increasing the concentration of thrombin in the reaction mixture or by reducing the pH.<sup>1</sup> In addition, Alexander<sup>1</sup> has shown that the plasma of a patient with congenital afibrinogenemia exhibited prolongation of the prothrombin time similar to that of normal plasma under the same conditions of storage.

The observed alteration in stored prothrombin-free plasma (human) appears to be related in part to the development of antithrombic activity possibly resulting from treatment with barium sulfate per se, since untreated oxalated and citrated plasmas containing prothrombin do not develop antithrombin activity at the same rate of speed or to the same extent when stored at  $30^{\circ}\text{C}$ . The observed prolongation of the prothrombin time and apparent antithrombic activity have been shown to occur concomitantly with a rise in pH by Tantum and Wetzel.<sup>14</sup> They have reported a prompt reduction in antithrombic activity in prothrombin-free plasma following the lowering of the pH to 7.6 to 7.8. In addition to this observation, the formation of fibrin by the action of thrombin on fibrinogen has been shown, by Morrison,<sup>8</sup> to be intimately concerned with the pH of the reaction mixture. Below a pH of 5.7 no fibrin will form, while between a pH of 6 and 7 there is a noticeable increase in the rate of reaction as the pH rises. As the pH is further increased above 7, there is less obvious increase in the rate of reaction.<sup>2</sup>

From the available data reported here, it is impossible to conclude that pH changes alone are responsible for the antithrombic activity since prothrombin-free plasma (human) stored at  $-20^{\circ}\text{C}$  in the frozen state for twenty-four hours showed an increase in pH when thawed without antithrombic activity. This is further borne out by the observations of Tantum and Wetzel<sup>15</sup> who have shown that the antithrombic activity can be abolished without lowering the pH of the reaction mixture by the addition of protamine sulfate. They have suggested from this that stored prothrombin-free plasma may have an increased heparin content. For clarification of the relationship between pH changes and antithrombic activity further work is required.

It has been suggested<sup>15</sup> that the observed antithrombic activity of stored prothrombin-free plasma is responsible for prolongation of the prothrombin time of fresh plasma diluted with it. This would seem true only in part since other factors also are involved. If the pH of nonlyophilized stored prothrombin-free plasma (human) is adjusted to 7.4 and the plasma tested for labile factor activity (Quick),<sup>13</sup> there is found a progressive reduction in the amount of this substance as the duration of storage lengthens even though a pH of 7.4 is maintained. The lower the temperature of storage, the less tendency there is for such a reduction to occur, as noted elsewhere.<sup>12</sup> There would thus appear to be



at least two factors responsible for the prolongation of the prothrombin time of fresh normal plasma (citrate) diluted with stored nonlyophilized prothrombin free plasma. Initially, an apparent antithrombin develops causing mild delay in the speed of coagulation. After a relatively short period of storage at 37.5° C and 30° C there occurs gradual inactivation of the "prothrombin accelerators"<sup>13, 10</sup> which results in further slowing of the speed of coagulation. The additive effect of these two changes presumably results in a significant prolongation of the coagulation time to a limit after which time the antithrombic effect becomes stabilized as does the pH. The 'prothrombin accelerators' finally become completely inactivated.

By lyophilizing prothrombin free normal plasma (human) as outlined by Florsdorf and Mudd<sup>5</sup> observing then precautions to establish the proper relationship between total volume of material and available surface during the process so as to insure proper lyophilization, it is possible to maintain such material in stable form for many weeks. By reconstituting it to a pH of 7.3 to 7.4 a diluent is obtained that is quite active and which possesses no antithrombic activity or additional loss of prothrombin accelerators when used promptly. Other observers<sup>15</sup> have reported increases in pH and antithrombic activity to occur in lyophilized prothrombin free plasma. Such findings have not been observed here over a period of storage of ten to twenty weeks. Why this discrepancy exists is unknown since we have observed the same results of stability when using unbuffered distilled water for reconstitution of the lyophilized prothrombin free plasma. It is possible that the cause of such altered activity in the material studied by others<sup>15</sup> is incomplete lyophilization.

Using barium sulfate as an adsorbing agent of prothrombin there also occurs a reduction in certain plasma components that are intimately concerned with the speed of the coagulation reaction. To what extent barium sulfate removes the "accelerator substances" is unknown but it is reported that approximately 50 per cent of Owren's Factor V is removed although the amount of BaSO<sub>4</sub> required to effect this removal is not stated.<sup>10</sup> The difficulty of simultaneously depleting plasma of its prothrombin and certain of the accelerator substances can be eliminated by using as a diluent bovine plasma treated with barium sulfate. Owren<sup>10</sup> and Seegers<sup>9</sup> both have shown that bovine plasma contains much greater quantities of their factors than does human plasma. Because of this observation Owren<sup>10</sup> advocates the use of prothrombin free bovine plasma as a diluent of human plasma so as to insure an excess of Factor V. Since it appears likely<sup>3</sup> that Factor V of Owren<sup>10</sup> plasma accelerator globulin of Seegers<sup>10</sup> and labile factor of Quick<sup>13</sup> are the same activities it is possible to have excesses of these activities as well as an excess of plasma thromboplastin and fibrinogen in the dilution method of Rosenfield and Tuft for the determination of prothrombin concentration by using prothrombin free bovine plasma as a diluent of fresh citrated human plasma.

In the lyophilized state prothrombin free bovine plasma maintains unaltered activity as a reconstituted diluent of fresh human citrated plasma and no antithrombic activity or pH changes were observed over a storage period of twenty

weeks. Because of the greater "prothrombin accelerator" activity in bovine plasma, it is necessary to establish a prothrombin time-concentration curve using prothrombin-free bovine plasma as a diluent. The shorter prothrombin time obtained at a 50 per cent concentration of prothrombin (see Fig 1) using prothrombin-free bovine plasma as a diluent is most likely due to the greater "prothrombin accelerator" activity of this diluent.

By lyophilization of prothrombin-free normal human or bovine plasma, there is thus placed within easy reach of many laboratories and physicians a reagent whereby more accurate determinations of prothrombin can be accomplished. With proper reconstitution of a stable (lyophilized) thromboplastin and a stable (lyophilized) prothrombin-free diluent of fresh normal citrated plasma, prothrombin determinations can be performed reproducibly.

#### SUMMARY AND CONCLUSIONS

Experimental data are presented showing the great instability of normal human plasma rendered prothrombin-free by treatment with barium sulfate and stored at various temperatures in the liquid state. Such instability is shown to result in prolongation of the prothrombin time of mixtures of fresh normal citrated plasma and stored prothrombin-free plasma. This instability is thought to be due to the occurrence at  $37.5^{\circ}\text{C}$  and  $30^{\circ}\text{C}$  of antithrombic activity as well as inactivation of the "prothrombin accelerators." The mechanism of development of antithrombic activity is not well understood. Lyophilized prothrombin-free human and bovine plasma stored at  $-20^{\circ}\text{C}$  for ten and twenty weeks respectively are shown to possess unaltered activity as diluents when reconstituted with buffered distilled water and used immediately. A discussion is given of the advantages of using a dilution method for the determination of prothrombin concentration, employing prothrombin-free bovine plasma as a diluent of fresh normal citrated plasma. Prothrombin time-concentration curves using three different diluents are included and the need for selection of a proper diluent is obvious. It is recommended that lyophilized prothrombin-free bovine plasma, reconstituted with buffered distilled water, be used as the diluent of fresh normal citrated plasma in the determination of prothrombin concentration, as a means of supplying an excess of certain "prothrombin accelerators" to a coagulation system in which prothrombin thus remains as the only variable.

It is concluded that prothrombin-free human and bovine plasma (1) maintain stability when properly lyophilized and stored at  $-20^{\circ}\text{C}$  for ten and twenty weeks respectively, and (2) when reconstituted to pH 7.3 are ideal diluents of fresh plasma for the determination of prothrombin concentration.

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## BIOLOGIC STUDIES WITH ARSENIC<sup>76</sup>

### III THE EFFECT OF ARSENIC<sup>76</sup> UPON THE CLINICAL COURSE OF PATIENTS WITH TUMORS OF THE HEMATOPOIETIC TISSUES

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#### INTRODUCTION

SINCE both stable arsenic and radiation have been used independently in the treatment of tumors of the hematopoietic organs it was thought that it might be advantageous to utilize radioarsenic ( $\text{As}^{76}$ ), a substance which is metabolized like stable arsenic, exerts a similar chemotherapeutic effect, and is at the same time a source of radiation

$\text{As}^{76}$ , which has a half-life of 26.8 hours and which emits energetic beta and gamma rays, was prepared by pile irradiation of arsenic trioxide, and later in higher specific activity by pile irradiation of cacodylic acid. It was injected intravenously as sodium arsenite. The stable arsenic content usually varied from 3 to 10 mg of arsenic per single injection of 1 to 80 mc of  $\text{As}^{76}$ . Cacodylic acid, as obtained after pile irradiation at the Argonne National Laboratory, is prepared for injection by remote control from behind lead shields because of its penetrating gamma radiations. The details of the preparation of  $\text{As}^{76}$  and its metabolism have been given in two preceding reports.<sup>1,2</sup>

Arsenic is distributed rapidly to all tissues after intravenous injection. Highest levels are reached within twelve to twenty-four hours in the spleen, liver, and kidney. Fifty per cent is excreted within seventy-two hours, primarily by the kidney. It is estimated that 1 mc of  $\text{As}^{76}$  will deliver about 1 r of total body radiation.

#### METHODS

Twenty-four patients with tumors of the hematopoietic tissues, two with polycythemia rubra vera, and one with a metastatic carcinoma form the basis of this study. The diagnosis was substantiated microscopically in every case. Many of the patients had repeated biopsies of bone marrow, liver, lymph node, and spleen, which will be made the subject of a future report (Table I).

The first few patients in this study were treated with extremely small amounts of  $\text{As}^{76}$  as far as specific radiation effect was concerned, although receiving at the same time enough stable arsenic probably to exert a mild chemotherapeutic effect. The first doses attempted were about 0.5 to 2.0 mc, but within a short period of time this was increased, partly because of experience with the inadequacy of these doses and partly because of the availability of arsenic with a higher specific radioactivity. As much as 90 mc with about 5 mg of stable arsenic were administered in a single injection to the recently treated patient. In addition, each patient received such supplemental treatment as transfusions, antibiotics, Digiloid and mercurials, and toluidine blue or protamine sulfate as indicated.

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TABLE I DIAGNOSIS DOSE AND RESPONSE TO TREATMENT WITH AS<sup>76</sup>

DIAGNOSIS	CASE	MILLICURIES OF AS <sup>76</sup>	RESULTS
Acute leucemia	1	24 on 10/24/47 60 on 11/12/47	No remission, died of hemorrhage
	2	69	No remission died of hemorrhage
Subacute myelogenous leucemia	3	135 on 10/3/47 12 on 10/10/47 45 on 1/31/48 47 on 5/14/48	Three remissions of 2 to 3 mo each
	4	17	Inadequate dose died of intracranial hemorrhage
	5	90	Remission of 1 wk then steady downhill course
	6	30	Symptomatic improvement for 2 wk until death from cerebral hemorrhage
Chronic myelogenous leucemia	7	30 on 3/31/47 34 on 2/1/49	First remission of 5 mo, second of 2 mo
	8	60	Good remission for 4 mo
	9	40	Good remission for 19 mo
	10	32 on 6/11/48	Six week remission on inadequate dose, second remission of 2 mo
		39 on 1/28/49	
	11	69 on 2/4/49 36 on 2/11/49	Symptomatic improvement 1 mo
Subacute lymphatic leucemia	12	36 on 1/30/48	Little improvement concurrent hemolytic anemia
	13	39 on 2/6/48 60	Remission of 2½ mo but had series of pyogenic abscesses
	14*	14	Remission of 1 mo then died of hemorrhage
	15	72	Remission of 1 mo then died of generalized tuberculosis
Chronic lymphatic leucemia	16	38	No response in severe mental depression at time of treatment
	17	38	Died of cerebral hemorrhage after 4 wk of symptomatic improvement
	18	80	Two week remission died of pericardial effusion
	19	76	Five month remission until death from congestive heart disease
Polycythemia rubra vera	20	45	Three month remission
Multiple myeloma	21	15 on 8/28/47	Six month remission (unusually benign type of case)
	22	12 on 9/3/47 88	Seven month remission
Mycosis fungoides	23	80 on 9/10/48 80 on 9/24/48	No response later responded to x ray
Metastatic carcinoma primary in stomach	24	80	Decrease in size of liver but progressive downhill course

Referred by Dr Arvid Johnston Rockford Ill

## RESULTS

*Hodgkin's Disease*—Each of four patients with Hodgkin's disease received from 10 to 130 mc of As<sup>76</sup> and up to 52 mg of stable arsenic in divided doses. The longest remission two months was obtained in a patient receiving 52 mg of stable arsenic and 136 mc of As<sup>76</sup>. However it was felt that the amount of stable arsenic was such as to be sufficient in itself to produce a remission and that the As<sup>76</sup> had not been given an adequate trial in this group.

*Acute Leucemia*—Both patients experienced a sharp drop in the white count and platelets but no significant change in the differential count (Fig 1) Case 1 developed a severe hemorrhagic process and Case 2 had a terminal rise in the white count to 180,000. Both patients had an intractable anemia which failed to respond to therapy of any nature including massive transfusions.

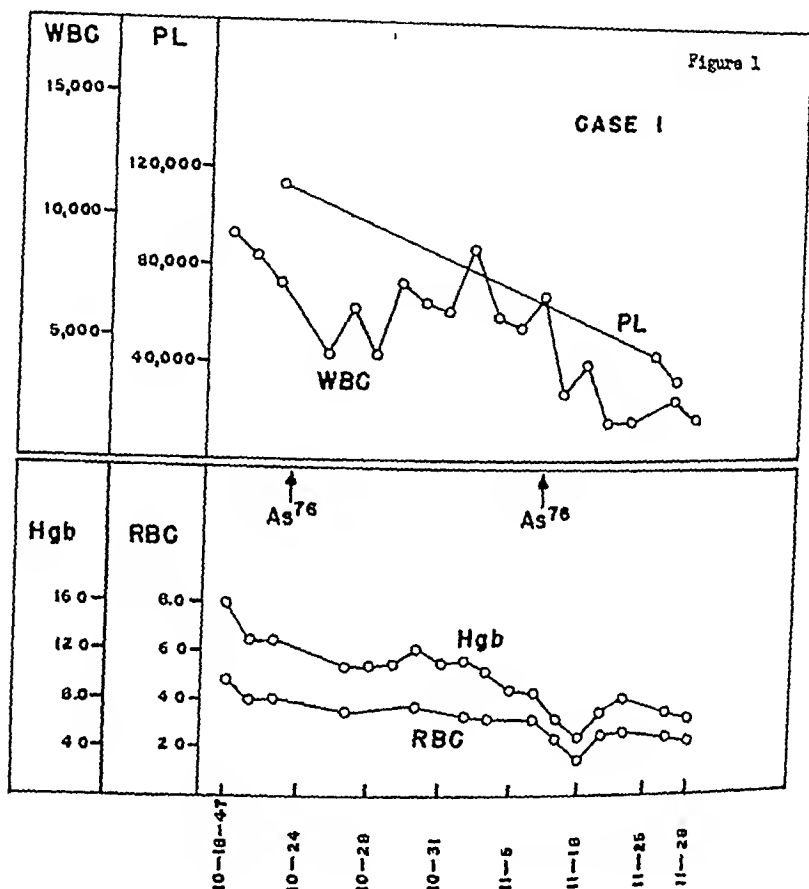
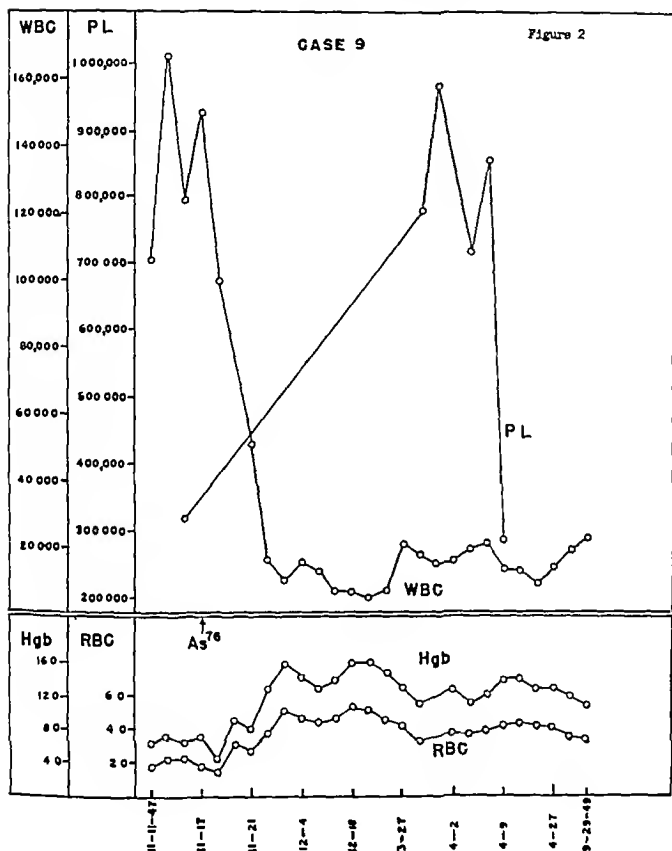


Fig 1

It is clear that these two patients failed to show any better response to As<sup>76</sup> than has been noted in the past to urethane,<sup>3</sup> P<sup>32</sup>,<sup>4</sup> nitrogen mustard,<sup>5</sup> or x-ray.<sup>10</sup> It is probable that Amnopterin<sup>11, 12</sup> will have more to offer than As<sup>76</sup> in the treatment of acute leucemia.

*Subacute Myelogenous Leucemia*—Case 4 did not receive enough As<sup>76</sup> to exert a therapeutic effect. Case 5 had an extremely short remission but the patient was in very poor condition when treatment was begun. If the result obtained in Case 3, in which there were three remissions varying from about four months to two months in spite of an intractable osteomyelitis, can be duplicated in other cases of subacute myelogenous leucemia, it would appear that As<sup>76</sup> may be about as effective as Amnopterin<sup>12</sup> and superior to the more common therapeutic agents<sup>1-10, 13</sup> and radiosodium<sup>14</sup> in the treatment of this condition.



Fig

*Chronic Myelogenous Leucemia*—Four (Cases 6, 8, 9, and 11) of the six patients were previously untreated and one (Case 7) had one course of methane and should also be classified as in early case. Four patients responded with remissions from four to sixteen months in duration (Fig 2) and two (Cases 7 and 10) responded in a similar manner to a second course of As<sup>76</sup>. The other untreated patient (Case 11) had only slight symptomatic improvement. The sixth patient (Case 6) refractory to x-ray therapy, died

of a cerebrovascular accident. However, his anemia failed to respond to transfusions following  $As^{76}$  and he may be classified as a therapeutic failure as is usually the result in patients of this type.<sup>4</sup>

Chronic myelogenous leukemia is a disease with great variations in its course. Patients have been known not to develop any subjective symptoms for as long as five years after the diagnosis was established.<sup>10, 16</sup> However, only a minority (16 per cent) of patients have spontaneous remissions once the disease produces anemia or subjective symptoms.<sup>10</sup> Therefore, any good result obtained in patients who were treated merely on the basis of a positive diagnosis may be ascribed to the benign nature of the disease rather than to the direct effect of therapy.

With this reservation in mind it would appear that even the greater differences in response reported in previously untreated patients following x-ray,<sup>16, 18</sup>  $P^{32}$ ,<sup>4, 5, 18</sup> methane,<sup>3, 19</sup> Fowler's solution,<sup>20</sup> and  $Na^{24}$ <sup>14</sup> may well be due to variation in the biologic nature of each patient's disease. It would, therefore, appear that remissions of four to sixteen months in our untreated cases represent no better than an average response (Fig. 2). Like other generalized methods of therapy,  $As^{76}$  is not as efficacious in reducing the size of a huge spleen as local x-ray therapy,<sup>4</sup> but like  $P^{32}$ ,<sup>4, 5, 18</sup> and  $Na^{24}$ <sup>14</sup> it rarely produces any toxic symptoms.

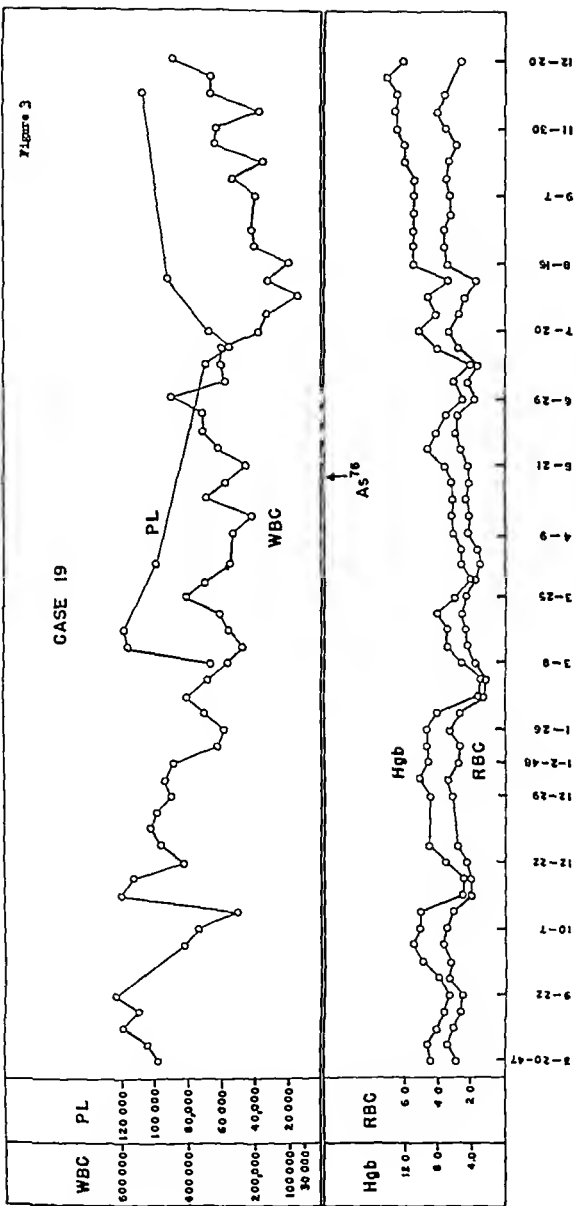
*Subacute Lymphatic Leucemia*—Two of the four patients (Cases 13 and 14) were previously untreated and had symptoms of comparatively short duration. One patient, Case 15, had received x-ray therapy with very little benefit and one, Case 12, had received methane without improvement. Three (Cases 13, 14, and 15) responded to therapy with an improvement in appetite, decrease or absence of fever, decrease in lymphadenopathy, and lowering of the white count for about two months. However, two (Case 13 and 15) of these three experienced the onset of a severe infection, furunculosis in one, tuberculosis in the other.

The fourth patient (Case 12) had a hematologic response with a drop in white count and a decrease in fever, but because of an associated hemolytic anemia he continued as an invalid until his death one year later following splenectomy in another institution.

These results are, in general, comparable with those reported in the literature following Aminopterin.<sup>12</sup> Possibly subacute lymphatic leucemia may show a better response to  $As^{76}$  than that reported after  $P^{32}$ ,<sup>4, 5</sup> methane,<sup>3, 19</sup> or nitrogen mustard.<sup>13</sup> The occurrence of two severe infections (Cases 13 and 15) at the time of greatest depression of the white count should warn against the use of  $As^{76}$  in the presence of any generalized infection, especially tuberculosis. We have been impressed by the occurrence of a flare up in cases of previously quiescent tuberculosis following generalized cytotoxic therapy such as  $As^{76}$  and nitrogen mustard in the treatment of leucemias and lymphomas.<sup>1</sup>

*Chronic Lymphatic Leucemia*—Four patients were treated with fairly adequate amounts of  $As^{76}$ . Only one (Case 16) was previously untreated. He was in a severe mental depression and failed to respond to therapy. Two patients (Cases 17 and 18), each of whom had been subjected to extensive x-ray





therapy in the past, succumbed to complicating diseases. A spectacular result was obtained in the last patient (Case 19) since an intractable anemia was relieved and massive lymphadenopathy and splenomegaly were decreased (Fig 3).

Our results, in general, are not as favorable as those reported after x-ray<sup>16-22</sup> or  $P^{32}$ ,<sup>4</sup> nitrogen mustard,<sup>7</sup> urethane<sup>3, 19</sup> or  $Na^{24}$ .<sup>14</sup> Particularly discouraging was the complete failure in the patient, Case 16, who was previously untreated. However, Reinhard<sup>4</sup> has encountered a similar result after  $P^{32}$ , as we have after nitrogen mustard.<sup>21</sup> The response of Case 19, who had remained severely anemic in spite of massive transfusions and several types of therapy, was most encouraging since this is the type of case which is notoriously difficult to manage.

*Polycythemia Rubra Vera*—The response of Case 20 was fairly satisfactory and also was qualitatively similar to that described after  $P^{32}$ .<sup>4, 23</sup> However, much longer remissions are not at all unusual after  $P^{32}$  so that  $As^{76}$  has little to offer that is superior to the results already achieved by  $P^{32}$ . Another patient was treated with a total of 24 mc of  $As^{76}$  and phlebotomies. Since this amount of  $As^{76}$  is too small to be effective, the brief remission probably was due to the phlebotomies.

*Multiple Myeloma*—This disease is almost always characterized by a rapidly progressive downhill course with unrelenting pain in untreated cases, although occasional chronic cases such as Case 21 have been recognized.<sup>4</sup> Remissions similar in extent to that experienced in Case 22 have been reported after urethane<sup>23</sup> and Stilbamidine.<sup>26, 27</sup>

*Mycosis Fungoides*—In many respects the therapeutic results in this disease are similar to those achieved in Hodgkin's disease. In Case 23, treated four months before death, it was quite clear that the skin and pulmonary lesions were still in part amenable to local x-ray therapy after they had failed to respond to  $As^{76}$  or nitrogen mustard.

*Metastatic Carcinoma*—The results in Case 24, in which the patient had a carcinoma of the stomach, are difficult to interpret. Carcinoma of the stomach may occasionally give rise to masses that respond to x-ray therapy by a decrease in size as did this patient's liver metastases. There was little else in the patient's course to suggest that  $As^{76}$  would prove emetative or even beneficial in carcinoma of the stomach.

#### DISCUSSION

It appears, therefore, that  $As^{76}$  resembles numerous other therapeutic agents such as x-ray, urethane, nitrogen mustard, radiophosphorus, radio sodium, and Fowler's solution in that it is capable of producing a remission in certain of the more chronic and benign tumors of the hematopoietic tissues and that it is not a cure for these diseases. The more acute forms of these diseases, with the possible exception of subacute leukemia, do not seem particularly amenable to  $As^{76}$  therapy.

In evaluating the clinical usefulness of  $As^{76}$  one is forced to consider whether it is more effective than previously used materials in producing a re-

mission, whether it will produce a remission where other agents have failed, and whether the advantages if any are sufficiently great to warrant using a substance that is so difficult and expensive to prepare and so dangerous to handle. Unfortunately, because of numerous factors, such as intercurrent disease, variations in the clinical course of patients with the same disease, duration of disease previous therapy supplemental therapy, and rest and psychic factors it is very difficult to comprise any series of patients. One cannot but be impressed by the occasional patient who responds to therapy in a manner that is entirely unexpected and inconsistent with the usual response characteristic of patients with that disease.

The criteria for evaluating a clinical remission are not similar. For example, depression of the white count has frequently been used as a means of estimating the response to therapy. However, particularly in acute leucemia and the terminal stages of chronic leucemia the height of the white count may bear no relation to such response. In this study we have followed the blood counts carefully and have noted a depression of the white count varying from 20 per cent to 80 per cent of the pretreatment figure in almost every patient treated without an invariable improvement in the clinical condition of the patient. Consequently the white counts have not been reported in detail. There has been no marked tendency of the peripheral blood differential count to return to normal following treatment except in a few cases of chronic myelogenous leucemia. An alleviation of the anemia is generally acceptable as an indication of a good therapeutic response. Since practically every one of the patients in this study received numerous transfusions this criterion was rendered unreliable.

In general we have felt that the ability of the patient to live a more comfortable and more useful life was the best indication of a response to therapy.

On this basis our results seem to have been unusually good in a single case of chronic lymphatic leucemia characterized by severe anemia possibly favorable in subacute lymphatic leucemia and worthy of further evaluation in multiple myeloma. In the other diseases treated the recognized agents such as X-ray, P<sup>32</sup> methane and nitrogen mustard which are more readily available, less expensive, and less hazardous will give similar if not superior clinical results.

It is of importance to note that we have not yet attained a maximum safe clinical dosage level of As<sup>6</sup> nor have we fully explored the combination of stable and radioarsenic or the effect of repeated small doses of As<sup>76</sup>. It is possible that when these are attained a better clinical response may be achieved. However, the problems of handling the amounts of As<sup>76</sup> we have used are so great that it hardly seems practical or worth while to attempt to use larger quantities of As<sup>76</sup> on the basis of the response of the patients in this study.

#### CONCLUSIONS

1 Twenty four patients with tumors of the hematopoietic tissues, two with polycythemia rubra vera and one with metastatic carcinoma have been treated with intravenous injections of radioarsenic (As<sup>76</sup>)

2 This radioisotope, under the conditions used in this study, appears to offer no advantages over the types of therapy already in use for these conditions

We wish to thank Mr Howard Ducoff and Mr Robert L Straube of the Argonne National Laboratory for their cooperation in the preparation of the  $As^{66}$  used in this study

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# THE USE OF RADIOACTIVE SILVER FOR THE DETECTION OF ABSCESES AND TUMORS

## I THE CONCENTRATION OF $\text{Ag}^{111}$ IN SPONTANEOUS AND EXPERIMENTALLY INDUCED ABSCESES

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IN A recent communication from this laboratory<sup>1</sup> it was pointed out that tracer doses of the long half-life silver isotope,  $\text{Ag}^{108-110}$  (half-life 225 days) could be caused to concentrate at particular localities in the bodies of albino rats by the simple expedient of inducing infection (experimentally) in the form of abscesses. It also appeared that the radioactive isotope concentrated itself in areas of spontaneous infection. It seems possible that if the short half life radioactive isotope,  $\text{Ag}^{111}$  (half-life 7.5 days) could be concentrated at or near the site of a lesion in sufficient amounts it might prove of value in therapy of tumors in human beings.

It is clear that if radiosilver can be caused to concentrate in abscesses, induced by injection of a suspension of bacteria, or in areas of spontaneous infection the procedure may become a very valuable method for the detection or location of hidden or obscure abscesses. The existence of the 8.2 day half life  $\text{Ag}^{108}$  and the newly developed, highly sensitive, directional Geiger-Muller counters make the procedure ideal as a tool for diagnosis in the patient with an obscure focus of infection.\*

In the part which follows it will be shown that the  $\text{Ag}^{111}$  behaves similarly to the  $\text{Ag}^{108-110}$  (as would be expected from the electron configurations), viz it concentrates in spontaneously or experimentally induced infected areas. The directional Geiger-Muller counter was not used in these experiments with  $\text{Ag}^{111}$ . It was necessary to conduct the assay through actual ashing of the tissues and chemical isolation of the radiosilver.

### EXPERIMENTAL

The  $\text{Ag}^{111}$  was separated from the irradiated palladium target obtained from the pile at Oak Ridge by a procedure developed in the Cancer Research Laboratory of this Institution by Rouser and Hahn.<sup>2</sup>

The test organism used was a strain of *Streptococcus hemolyticus* isolated from the throat of a hospital patient. The organism was used after sixteen hours of incubation in proteose peptone broth at 37° C. One tenth of a milliliter of the undiluted culture was injected into the right legs of albino rats and forty eight hours later 0.5 milliliter of

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\*The  $\text{Ag}^{108}$  isotope is the isotope of choice for this purpose since it produces gamma rays and unlike  $\text{Ag}^{111}$  which is a pure  $\beta$ -ray emitter should be amenable to external survey. Pure  $\beta$ -ray emitters are useless for this purpose since their particles travel only short distances. The 225 day half-life isotope  $\text{Ag}^{108-110}$  while it emits gamma rays and thus would be detectable in external surveys is excluded for this purpose on account of the danger of prolonged irradiation resulting from its long half-life. This would be particularly hazardous if for some reason the isotope is not promptly excreted. On the other hand  $\text{Ag}^{111}$  has a relatively short half-life of 7.5 days.

$Ag^{111}$  in the form of the nitrate was injected either into the same leg or into the left one. Three days after injection of the isotope the animals were sacrificed and the legs, kidneys, livers, lungs, hearts and testes or ovaries were assayed and the silver was isolated and subjected to radioassay as described previously.<sup>1</sup> The results with five animals are given in Table I. Each tissue was subjected to bacteriologic examination before assay.

TABLE I. DISTRIBUTION OF  $Ag^{111}$  IN VARIOUS TISSUES 72 HOURS FOLLOWING ITS INJECTION IN LEFT OR RIGHT LEG IN THE PRESENCE OR ABSENCE OF INFECTION, EXPERIMENTALLY INDUCED IN THE LEFT LEG  
(DOSE 0.5 CC WITH  $3.62 \times 10^4$  COUNTS PER MINUTE)

RAT	RIGHT LEG	LEFT LEG	KIDNEY	LIVER	LUNG	HEART	TESTES OR OVARY	RIGHT LEG	LEFT LEG
1		Isotope	310	820	85	55	780	1 220	2 620
2		Organism							
3	Isotope	Organism	400	862	75	40	18	1 065	3 220
4	Isotope	Organism	492	1 690*	91	80	20	1 620	4 000
5	Isotope	Organism	472	1 261	97	49	27	1 026	3 221
		Isotope	472	1 097	101	52	23	1 262	1 415

Bacteriologic test positive indicating infection

It was of interest that the concentrations of radiosilver were uniformly high in livers. To confirm this observation three additional animals were given injections of  $Ag^{111}$  alone and the concentrations of the isotope in kidney, liver, lung, heart and testes or ovaries were determined. The results are shown in Table II where it is to be seen again that the livers have a uniformly high count.

TABLE II. DISTRIBUTION OF  $Ag^{111}$  IN VARIOUS TISSUES 72 HOURS AFTER ITS INJECTION INTO RIGHT LEG

RAT	RADIO-SILVER INJECTED I TO RIGHT LEG (COUNTS PER MIN.)				
	KIDNEY	LIVER	LUNG	HEART	TESTES OR OVARY
1	389	1 031	41	0	0
2	381	924	51	0	0
3	403	964	55	0	0
Control sample	36 147				

An additional three animals were studied for normal distribution of the isotope. In this instance the studies were extended to include as much of the muscle, bone, and blood as could be obtained and the feces and urine were collected. The stomachs and intestines were assayed together as well as the major portion of the skin. The heads and tails were not studied. The results are shown in Table III. Rat 1 must not be compared with Rats 2 and 3 since a massive infected area was found in the left leg (right leg injected with  $Ag^{111}$ ). The predominating organism in this infected organ was a strain of *Str. hemolyticus*. The tissue placed under the Geiger-Müller tube for radioassay was found to be highly radioactive.

# DISCUSSION OF RESULTS

The experimental findings given in Table I indicate in line with our previous results with the 225 day half life isotope, that  $Ag^{111}$  also concentrates in the tissue with the experimentally induced abscess. They demonstrate also that this will occur even if the isotope is injected into an organ quite remote from the focus of infection. The result with Rat 1, Table III, was extremely fortunate, though unexpected. The radiosilver was injected into the right leg. When the

TABLE III DISTRIBUTION OF  $\text{Ag}^{111}$  IN TISSUES OF THE ANIMAL BODY AND RECOVERY AFTER 72 HOURS

(HEADS AND TAILS NOT STUDIED RAT 1 WAS FOUND TO HAVE A MASSIVE INFECTED AREA IN THE LEFT LEG)

TISSUE	RAT 1 (COUNTS/MIN)	RAT 2 (COUNTS/MIN)	RAT 3 (COUNTS/MIN)
Liver	65	72	82
Lungs	11		
Kidney	26		
Skin		3	11
Bone		10	7
Muscle	5,179	122	130
Feces	879	5,763	5,970
Urine			
Blood	39	22	30
Testes or ovaries			
Intestine and stomach	29	114	122
Heart		35	21
Per cent recovery	78.03	85.14	88.30
Control sample (counts/min)	7,981	7,212	7,212

animal was sacrificed a massive infection of the left leg was discovered. The high count in the muscle fraction for this animal was chiefly due to the silver concentrated in this area. This result emphasizes the probable value of the method, if employed, for detection of obscure or hidden abscesses.

The uniformly relatively high count in the livers of the animals studied deserves some comment. The counts in the infected tissue are some three to four times as great as those seen in the livers and thus would hardly be cause for confusion in the search for a focus of infection. These relatively high counts after three days are apparently related to excretion of the silver. The high counts seen in the feces, the absence of silver from the urine, and the relatively high values for liver lead to the conclusion that tracer doses of radiosilver are excreted by the liver, presumably by way of the bile, into the intestines and excreted in the feces. These results confirm those of Scott<sup>3</sup> recently available from the Atomic Energy Commission.

Previous attempts to develop procedures for the detection of abscesses have been made by Kroll, Strauss, and Necheles<sup>5,6</sup> and Strauss, Neuwelt, Rovner, and Necheles.<sup>4</sup> In a recent publication<sup>6</sup> these authors suggest the use of the bis-azo dye, disodium-1-amino-8-hydroxy-naphthalene-3,6-disulfonate (II acid) monobrominated with radioactive bromine for localization in experimentally induced abscesses in dogs. The preliminary experimental results reported here would suggest that the employment of radiosilver nitrate in tracer doses may be a more simple procedure.

These studies are being extended to animals with the turpentine sterile abscess and with tumors.

#### CONCLUSIONS

- 1 The radioactive  $\text{Ag}^{111}$  isotope concentrates in areas of infection.
- 2 This isotope is excreted by the liver into the intestine presumably by way of the bile and the feces.



3 Since both  $Ag^{108}$   $^{110}$  and  $Ag^{111}$  concentrate in areas of experimentally induced and spontaneous infections it is to be expected that other silver isotopes such as  $Ag^{106}$  for example, would behave similarly. Since  $Ag^{106}$  has a short half life (82 days) and also emits gamma rays and is thus amenable to external survey it is suggested that it might prove to be of value as a tool for the detection of obscure foci of infection in the animal body.

The senior author wishes to express his gratitude to Dr P F Hahn of the Cancer Research Laboratories of this College for his assistance in the initiation of the program of isotope research.

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# THE MINIMAL SODIUM DIET—A CONTROLLED STUDY OF ITS EFFECT UPON THE BLOOD PRESSURE OF AMBULATORY HYPERTENSIVE SUBJECTS

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## INTRODUCTION

DESPITE the extensive studies on the place of diet low in sodium chloride in the therapy of hypertension,<sup>1-5</sup> there is as yet no complete agreement as to the efficacy or explanation of this therapy. Recent re-examination of this subject might lead one to the conclusion that the administration of diets which are low in sodium is sometimes attended by a reduction in blood pressure.<sup>6-9</sup> This, it has been claimed,<sup>6-9</sup> is a reason for the success<sup>10</sup> of the low diet therapy. Dietary restriction of sodium (including the low diet) has again become widespread and is generally being applied in clinical practice without adequate control. Several carefully controlled studies have dealt with patients in the hospital after they have reached a stabilized, or nearly stabilized, blood pressure level and in whom a period of sodium restriction has then been alternated with a period of more normal sodium intake.<sup>11, 12, 13</sup> From these studies has come the observation that in some patients a fall in blood pressure may occur during a period of severe sodium restriction, but that this fall may be slight and may occur only when the twenty-four hour intake of sodium is kept extremely low.

It is not satisfactory to carry over the results of these studies by attempting to apply them generally to the clinical treatment of hypertension. First of all, a hospital environment is not the usual environment for a human being. The factors which may relate to the maintenance (and perhaps the establishment) of hypertension are considerably modified by the simple fact of being in a hospital as a patient. Therefore, a form of therapy which is ineffective or relatively ineffective in the hospital might conceivably be more effective when the patient is under "normal" living conditions.

Second, it is possible that severe sodium restriction might serve to reduce blood pressure in the hypertensive patient only under certain circumstances. For instance, sodium restriction may be unable to further lower a blood pressure that has come down under influence of other factors to a minimum or "base line." If the patient had been treated at a higher level of blood pressure than this "base line," an effect might have been noted, even though the therapy is ineffective in lowering the "floor" of blood pressure.

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Most important of all the administration of a diet which is rigidly restricted in any factor may influence the habits and reactions of the patient quite profoundly. In this manner it may produce physiologic changes unrelated to the removal of the particular agent under study. In order to control this most prominent feature of dietotherapy it is mandatory to administer a diet identical to the experimental diet in every way, except for the factor under study. Moreover this must be done in such a way that the subject remains unaware of the difference. In addition in any study where the interests, emphasis or bias of the investigator might modify the results he too, should not know whether his observations relate to the experimental or the control period.

Two recent studies have been reported on the effect of rigid sodium restriction upon ambulatory patients in which favorable results are claimed.<sup>7,8</sup> These reports do not include controls either of the diet in terms of the degree to which the diet was followed or of the factors in the treatment other than lack of sodium which might be responsible for the results.

#### PLAN OF STUDY

For these reasons a controlled study was planned in which all patients would be placed upon the same dietary regimen—one which would be rigidly restricted in sodium content to less than 200 mg of sodium per day. The experiment was divided into three periods of six weeks each. Schedules were prepared for twenty-four patients covering the administration of either sodium chloride 4 Gm per day or an identically designed lactose preparation to these patients during the three experimental periods. (A construction based upon three periods was chosen to make it impossible to be certain whether the medication was being changed or not at the conclusion of any one period.) Of the eight different combinations of periods possible these four were selected: 1 Lactose sodium, lactose 2 sodium lactose lactose 3 lactose lactose sodium 4 sodium lactose sodium.

A schedule was selected in random fashion by the pharmacist and assigned to each patient, the medication to be issued from the pharmacy according to this schedule and without knowledge of the patient or the clinic personnel. Although occasionally a patient had gastrointestinal distress which might suggest that he was receiving sodium chloride in the main filter was no indication as to which medication was being taken.

The subject was seen throughout by the same investigator, who, following a uniform technique each week recorded the blood pressure on one particular arm after the patient had rested in the recumbent position for at least five minutes averaging two or three determinations. The recommended technique for blood pressure measurement was followed.<sup>10</sup>

Adhering to the diet as planned was not an easy task. As a practical check, twenty-four hour urine samples were collected usually once a week for sodium analysis with creatinine analysis to establish the completeness of the sample. While urinary sodium will not parallel dietary sodium during retention in diuresis or other state of negative sodium balance, the length of the observational periods makes it unlikely that any significant storage or loss could occur and not be reflected by weight change.

The technician alone had access to the records of medication. Accordingly if the sodium excretion exceeded the estimated intake the doctor and dietitian could be so informed. The checking procedure resulted in some delay and this explains why a number of patients were permitted to continue the experiment although their sodium intake was so high that they

These were initially provided as gelatin capsules containing 0.5 Gm through the courtesy of Dr. L. L. Joslyn and the Abbott Laboratories, North Chicago, Ill. Subsequently specially coated sealed tablets were used obtained through the courtesy of Dr. K. G. Kohlschütter and Eli Lilly & Company, Indianapolis, Ind.

We are greatly indebted for the able cooperation of Mr. M. L. Jetterling and her staff.

cannot now be considered to have been on a rigid sodium restriction. While some patients will follow the diet with precision and have no difficulty in maintaining a urinary twenty-four hour sodium well below 300 mg, other patients cannot be made to understand, or do not want to accept, the limitation of a diet so constructed.

*Selection of Diets*—The diets were individually prescribed after interview. They attempted to meet the doctor's prescription as to caloric level (maintenance was desired) and as to protein content (usually as high as the patient would accept), to consider the patient's tastes, and still to have the calculated sodium content below 300 milligrams. A commercial preparation of salt depleted reconstituted milk\* was employed where indicated. Commercial salt substitutes were permitted if they contained no sodium†. Iron and vitamin deficiencies were corrected by medication where necessary. Water was allowed ad libitum, the sodium content being known or determined to be insignificant. In several instances "holidays" were permitted for Christmas or New Year's Day. Although these were followed by a mercurial diuretic and when necessary an additional urinary collection, relaxation to this degree was a mistake, at least in the handling of the experiment. We did feel at the time that it kept the cooperation of the subject. We were aware of the variation in sodium content of foods according to selection and preparation, as well as differences in values cited by different authorities<sup>17, 18</sup>.

*Selection of Patients*—Twenty-four patients were selected on the basis of the existence of definite diastolic hypertension (above 90 mm Hg) after several clinic visits. More than half of the patients had long records of previous attendance at the clinic. A group was chosen who expressed themselves as willing to follow the procedure with the understanding that they were participants in an experimental study. Since the experiment contained its own inherent control it was not thought necessary to observe these patients for any period on a normal diet prior to placing them on the experimental regime. In addition, a type of incomplete control was available in the records of those patients who had been attending the clinic previously, but in these cases blood pressures were not taken always in the same manner or by the same observer. A second type of control was offered in the several patients in whom diet was begun before the "special" medication was available, and these patients, therefore, had a period of sodium restriction during which time they were not given medication. In one or two instances after the experiment was completed and it was determined that the patient had not followed the diet correctly, a final experimental period was added during which no "special" medication was given and the patient was observed on the salt restriction alone. The ages of the patients were between 28 and 70. Nine were men. The etiology of hypertension was considered to be known in two cases, one of glomerulonephritis and one of pyelonephritis with nephrolithiasis. Two patients previously had undergone sympathectomy. Nine of the patients were in congestive heart failure, renal insufficiency, showed hypoproteinemia or lowered serum sodium or chloride concentrations, or had visible edema. Of the twenty-four subjects selected, twenty-one continued the study for at least two experimental periods and twenty of these for at least three periods. In some instances repeated determinations were made of the heart size by roentgenogram, the electrocardiogram, blood volume, and thiocyanate available space, but not enough information was obtained over the short period of this study to warrant any attempt at analysis. Other forms of medication which did not augment sodium intake (digitalis, phenobarbital, thiocyanate) were either discontinued at the outset, or continued unchanged during experimental and control periods.

## RESULTS

The entries, representing the average of the blood pressures recorded at each visit, were averaged for each experimental period. The values obtained are considered to represent the blood pressure for the subject under the conditions

\*Generous supplies of Lonalac were made available by Dr C. E. Pills of Mead Johnson & Company, Evansville, Ind.

†Neocortical Winthrop Chemical Company, Inc., New York, N. Y. Salt Control Dietetic Supply Co., Chicago, Ill.

‡Dr A. S. Alving and Dr R. M. Becker referred several of the patients to this study.

of the experiment (If, as has been claimed, an effect of sodium restriction may not appear for some time after the restriction of diet and may persist after discontinuance of the regime then the averages thus taken will diminish the apparent effect)

As a first analysis the blood pressures were averaged for all lactose periods for each patient and compared with the blood pressure obtained during the period or periods when sodium chloride was given. This construction assumes that the experiment was carried out as it had been planned originally. An analysis of the diastolic pressures according to this grouping fails to reveal any significant differences between intended experimental and intended control periods in the group as a whole. The subjects were then reclassified according to the results of the averaged twenty four hour urinary sodium assay. It is recognized that a single twenty four hour urinary collection may not represent the average of the excretion of sodium for a seven day period but by the same criterion the observations of blood pressure made one day in the week and only during one part of the day do not necessarily represent the average blood pressure of that patient over the entire week. With this realization, the experimental periods have been arranged under three headings. Where the twenty four hour urinary sodium samples averaged less than 0.5 Gm during a period the patient was considered to have been on rigid sodium restriction (A). When the average of the samples of twenty four hour urinary sodium was greater than 0.5 Gm but less than 1.05 Gm the degree of sodium intake was considered to have been intermediate (B). When the twenty four hour sodium samples averaged 1.05 grams or more the sodium intake was not considered restricted (C). On this basis only nine patients were observed to have actually maintained a rigid restriction for at least one complete period on an average basis. Two of these patients were on the restricted diet for two periods apiece. One patient was carried for only two periods and during both of these was on a restricted sodium intake.

There emerges therefore a group of eight patients who were under severe sodium restriction for at least one period and by comparison had at least one period of intermediate or relatively unrestricted intake of sodium. Table I indicates the average blood pressure during the experimental and the control periods for each of these subjects together with the experimental difference produced by the substitution of sodium chloride for lactose. The observed mean change in diastolic pressure was -4.7 mm of mercury with a standard deviation of 7.6. The mean difference between the average of systolic pressures was -8.7 mm of mercury with a standard deviation of 10.1.

It may be argued that these differences are entirely due to chance. As a test of the adequacy of our method of sampling blood pressures the observations on twenty one patients used in this study may be employed. While only eight of the group had been suitable to compare in experimental and control situations, twenty one of the subjects had been followed for at least *two* *11/2* periods. These may have been periods each of low sodium excretion, or periods

TABLE I AVERAGES OF BLOOD PRESSURES TAKEN DURING CONTRASTING SODIUM RESTRICTION PERIODS (DURING PERIODS A THE AVERAGE SODIUM EXCRETION WAS BELOW 0.5 GM/24 HR DURING B AND C IT WAS GREATER)

SUBJECT	SYSTOLIC (MM HG)			DIASTOLIC (MM HG)			NUMBER OF PERIODS	
	A	B OR C	DIFF	A	B OR C	DIFF	1ST	2ND
Sc	139.7	161.8	22.2	85.2	94.2	9.0	6	11
Ru	224.2	216.3	-7.9	113.7	105.7	-8.0	13	6
Br	183.1	198.3	15.1	92.0	93.6	1.6	7	7
Ca	173.4	178.4	4.9	99.3	102.1	2.8	7	11
Ti	179.3	187.5	8.1	107.3	112.7	5.4	6	13
Li	164.6	177.8	13.2	99.0	106.3	7.3	5	21
Li	163.5	179.8	16.2	90.5	109.0	18.5	2	8
Ma	160.4	158.3	-2.1	88.4	89.7	1.3	5	6
Average	173.5	182.3	8.7	96.9	101.7	4.7	6.4	10.1
s.d.			±10.1			±7.6		

of intermediate or unrestricted sodium excretion. Study of these twenty one pairs (Table II) indicates that two consecutive periods on a like regimen of sodium intake were associated with an average recorded difference in blood pressure averaging 0.6 mm of mercury diastolic and 1.1 mm of mercury systolic. Considerable variation in the differences observed between like periods in each subject was noted here and also was present in the eight subjects comprising the experimental group.

TABLE II AVERAGES OF BLOOD PRESSURES TAKEN DURING LIKE PERIODS OF SODIUM RESTRICTION

SUBJECT	SYSTOLIC (MM HG)			DIASTOLIC (MM HG)			NUMBER OF PERIODS	
	1ST	2ND	DIFF	1ST	2ND	DIFF	1ST	2ND
Sc	156.8	164.7	7.9	92.8	95.0	2.2	1	7
Ru	219.3	230.0	10.7	114.4	112.8	-1.6	7	6
Br	177.2	198.3	21.1	87.3	93.6	6.2	6	7
Ca	178.6	178.0	-0.6	101.9	102.5	0.6	7	1
Ti	198.3	184.2	-14.1	120.0	110.5	-9.5	3	10
Li	176.9	182.0	5.1	107.4	106.7	-0.7	7	7
La	183.0	174.3	-8.7	113.0	102.3	-10.7	5	3
Ma	156.5	162.0	5.5	87.0	95.0	8.0	4	2
Sw	193.1	194.8	1.7	110.6	113.8	3.3	7	6
Mc	209.4	213.5	4.1	110.0	110.6	0.6	5	8
Li	223.3	228.2	4.9	128.1	128.2	0.0	5	6
Gap	151.8	147.7	-4.1	79.2	78.8	-0.4	5	6
Ho	173.3	172.2	-1.1	99.2	96.2	-3.0	6	6
Ko	142.0	139.3	-2.7	96.8	100.3	3.5	5	5
Ce	205.3	208.0	2.7	112.8	106.6	-6.2	6	7
Pe	206.5	207.4	0.9	112.3	111.3	-1.0	6	5
St	182.3	175.8	-6.5	114.0	114.4	0.4	4	10
H1	241.0	232.6	-8.4	129.4	126.0	-3.4	7	5
Co	124.5	119.6	-4.9	77.7	77.6	-0.1	6	6
Ka	165.8	161.3	-4.5	90.6	87.8	-2.8	5	7
Gab	183.3	197.0	13.7	90.3	92.3	2.0	6	7
Average	183.3	184.3	1.1	103.6	103.0	-0.6	5.7	6.1
s.d.			±8.2			±4.5		

To determine the likelihood that the differences in the eight subjects of the experimental group might be due to chance alone, a "t" test of statistical reliability was applied\* using the formula

$$t_1 = \frac{\sum dp}{\sqrt{\sum p \sum r^2 / 1}},$$

\*We are indebted to Dr. L. J. Savage of the Department of Radiobiology, physics for direction advice and assistance with this analysis and also for well-tempered foil to our efforts.

where  $d$  = experimentally observed difference in blood pressure between periods of unlike sodium excretion

$p$  = weighting factor for  $d = \frac{n \times n'}{n + n'}$  in which  $n$  = the number of entries during the lactose period and  $n'$  = the number of entries during the sodium period

$r^2$  = the similarly weighted square of the difference between pairs of like periods, and

$a$  = the number of pairs of like periods or 21

This formula relates the experimental differences in the eight subjects to the variance of the blood pressure of the entire group of twenty one subjects. The  $t$  values obtained for 21 degrees of freedom were 2.93 for diastolic pressure and 3.17 for systolic pressure. On this basis the probability that a difference of the degree experimentally observed would occur by random variation alone is less than one in one hundred for both systolic and diastolic values.<sup>10</sup>

A tendency to lose weight was noted probably because of fluid loss and the difficulty of maintaining caloric intake. Every effort was made to keep the weight loss minimal. Some subjects even gained in weight and these were not exclusively the ones who were unable to restrict their sodium intake. The average weight loss was greater (2.0 Kg) in the twelve subjects whose sodium excretion was above 0.5 Gm than the weight loss (0.1 Kg) in the eight subjects who were able to rigidly restrict sodium. (Tables III and IV)

Generally the patients who could adhere to the diet claimed the most subjective benefit. Several could hardly be kept from continuing the regimen. One of these died abruptly three days after agreeing to liberalize her salt intake. The cause of death was presumed to be a cerebrovascular accident.

No evidence of undue salt deprivation was encountered. The study was not carried out during the summer months. It was the observers' impression that respiratory infections seem to be followed by undue degrees of asthenia. One patient developed pneumonia together with a condition resembling salt depletion. Her participation in the experiment was terminated and inquiry into the schedule revealed that she had been receiving supplemental salt. No abnormalities were noted in serum electrolytes in this or any other case.

Certain collateral information was obtained by analyzing the Clinic records of our subjects prior to and following the controlled experiment. Blood pressures taken by the same or by other observers and without uniform technique have been averaged for over three periods: (1) before 1943, (2) 1943 through 1945 and (3) 1946 and 1947 until the subject was selected for study. In certain instances the experiment was preceded also by a short period on diet alone and in some instances there was a further period of observation on unrestricted sodium intake at the conclusion of the study. The technique followed in these last two periods was essentially comparable to that used during the study except for the omission of the disguised sodium chloride or lactose.

TABLE III COMPARISON OF PREVIOUS BLOOD PRESSURES WITH VALUES OBTAINED DURING CONTROLLED ADMINISTRATION OF MINIMAL SODIUM DIET GROUP I

PA PAT.	AGE	SEX	WEIGHT CHANGE DURING EXPERIMENT (KG.)	BLOOD PRESSURE AVERAGES (MM. HG.)						AVERAGE URINARY SODIUM (GM./24 HR.)					
				VARIOUS RECORD			DIFF. ALONE*	PERIODS			DIET ALONE*	A	B	C	OFF DIET†
				BEFORE 1943	1946										
					1945	1947		A	B	C					
Sc	50	M	72.9 to 73.3	-	175	159	149	140	-	162	73	41	-	157	2.22
					95	91	84	85		94					
Ru	49	F	53 to 54.6	265	294	231	233	224	-	216	16	17	-	125	-
				135	143	143	126	114		106					
Bi	56	F	53.6 to 53.4	-	-	214	198	183	177	198	37	38	88	162	1.38
						100	96	92	87	93					
Cu	67	M	68.6 to 66.6	158	175	192	153	173	178	-	10	12	91	-	1.66
				94	110	113	90	99	102						
Th (Glomerulonephritis)	29	M	54.4 to 55.2	127	-	202	188	179	-	188	16	21	-	168	2.94
				84		135	122	107		113					
L1 (Pyelonephritis)	48	M	70.4 to 72.5	-	177	191	175	165	-	178	113	26	-	223	-
					111	112	114	99		106					
La	63	F	66.8 to 66.2	-	223	200	160	164	174	183	36	25	89	133	-
					134	115	94	91	102	113					
Ma	57	F	59.3 to 56.6	-	185	205	175	160	-	158	14	27	-	161	-
					97	110	100	88		90					
Average change from B				P of 1946 1947			Average								
				-44	2	-27	-32	-29	-22	39	26	89	161	2.05	
				-20	1	-10	-18	-15	-12						

\*1 to 1 week  
†1 to 7 weeks

\* 1 to 4 weeks  
† 1 to 7 weeks



GROUP II

IN- TENT	AGE	SEX	WEIGHT CHANGE DURING EXPERIMENT (KG.)	BLOOD PRESSURE AVERAGES (MM. HG.)						EXPERIMENT PERIODS						AVERAGE URINARY SODIUM (OM./24 HR.)						
				PREVIOUS RECORD			DIET			A			B			DIET ALONE	A	B	C			
				BLDGRE 1943	1943	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954							
Sw	54	F	70.9 73.6	105 117	186 121	185 111	-	-	-	-	193 112	-	-	-	-	-	-	-	1.70			
Mc	56	M	70.5 72.8	-	189 99	209 106	200 180	-	-	-	212 119	223 116	-	-	1.05	-	73	1.29	6.20			
La (Sympathetomizid)	44	F	03.0 53.0	-	229 131	230 129	-	-	-	213 126	225 128	226 128	-	-	-	-	85	1.76	3.41			
Gr	30	M	80.5	-	-	183 97	151 80	-	-	-	150 79	144 73	-	-	1.4	-	50	2.30	4.96			
Ho	54	F	64.9 63.0	195 103	201 110	212 117	172 109	-	-	174 101	173 98	197 110	-	-	0.3	-	104	1.87	4.16			
Ko	44	M	81.4 75.2	-	-	185 122	167 118	-	-	141 99	153 107	-	-	-	-	-	80	1.50	-			
Ce	49	F	74.3 76.7	213 117	220 123	226 123	173 100	-	-	208 107	205 113	-	-	25	-	10	1.45	-				
Pe (Sympathetomizid)	53	F	89.8 64	210 120	227 124	232 130	199 108	-	-	-	208 111	210 108	-	-	1.09	-	-	2.44	4.40			
St	51	F	70.7 71.2	-	-	187 121	162 108	-	-	-	180 110	190 121	-	-	34	-	-	1.81	3.06			
Hi	33	F	54.0 52.0	-	-	270 142	-	-	-	-	234 127	232 119	-	-	-	-	-	1.51	-			
Co		M	72.1 73	150 95	129 87	153 101	119 70	-	-	-	124 79	123 77	-	-	81	-	-	1.63	99			
Kn	50	F	61.5 66.9	-	-	174 99	166 99	-	-	-	164 89	153 82	-	-	1.43	-	-	1.74	1.23			
Average change				-2.0	Average change from B P of 1946 1947												Average					
				-1.4	-9	-7	-	-	-	-23	-19	-17	-	-	72	-	84	1.75	3.56			
				-5	-3	-14	-	-	-	-12	-11	-12	-	-	-	-	-	-	-			
				GROUP III																		
Gu	30	F	-	-	-	243 108	194 98	191 91	-	-	-	-	-	-	13	10	05	-	-			
				1 to 4 weeks																		
				11 to 8 weeks																		

The average results of this evaluation are recorded in Tables III and IV. For comparison, the experimental data are entered according to the level of urinary sodium as A, B, or C. The cases are arranged into three groups. The first group (Table III) includes the eight patients who completed at least an A and a B or C period. The second group (Table IV) includes twelve patients with no A period, and the third group consists of a single patient with A periods alone. The change in blood pressure in the first two groups is averaged, using the value for 1946 and 1947 as a base. The averaged sodium excretion also is shown for the experimental periods. The experiment in the third group was terminated by intercurrent illness. The comparison reveals an apparent fall in blood pressure in all groups during the periods of study. This fall is of a magnitude much greater than that experienced within the controlled portion of the experiment. For the study periods of lowest averaged blood pressure, in the eight experimental subjects used for evaluation of sodium restriction the average fall in systolic pressure was 30 mm. of mercury and in diastolic pressure 20 mm. of mercury. A similar comparison for the group of patients who did not follow a period of strict sodium limitation as defined shows an average maximum fall of 28 mm. of mercury systolic, and 16 mm. diastolic. There is a general tendency for the pressure to vary with the salt excretion. (It must be recalled that periods A, B, and C were not necessarily consecutive in any patient.) However, the sodium excretion after termination of the experiment was comparable to that of an unrestricted diet and, nevertheless, blood pressure average remained below the 1946-1947 level for both groups.

#### DISCUSSION

The average of the differences in blood pressure which can be ascribed to sodium restriction alone under the conditions of the study is shown to be quite small, 9 mm. systolic and 5 mm. diastolic. It should not be inferred from this that a regime of sodium restriction is ineffective or unwarranted therapy in uncomplicated hypertensive vascular or cardiovascular disease. Two important considerations should be emphasized in this regard.

(1) While the average response in the group of patients studied is small, certain individuals showed a greater response than others. Patient Ia, on the controlled portion of the study, evidenced a fall in diastolic pressure of 19 mm. of mercury. A fall of between 12 and 22 mm. of mercury in systolic pressure was evidenced in four cases. It is probable that there are individual differences in responsiveness among patients. Perhaps, also, the degree of response to sodium restriction is modified by other factors not herein controlled. It is to be emphasized that there may be different varieties of the disorder which is called "essential" hypertension in our ignorance of its homogeneity or etiology. When a large number of patients has been examined and the responsiveness of these patients to variation in sodium intake alone has been analyzed it may be possible to group the type of patient who responds into a separate category. The size of the present study does not permit deductions of this nature. Nor can we identify our responsive patients as being of the "pseudo Cushing's" type reported by Schroeder.<sup>12</sup>

(2) It will be noted by comparing the collaterally obtained information with that obtained during the experimental part of the study that all patients even those who responded very little to restriction of sodium alone had a reduction in blood pressure over the period encompassed by the study. This maximum change in pressure is the figure that would be obtained from an entirely "clinical" evaluation of these patients and represents the overall effect of all procedures used plus the spontaneous variations in blood

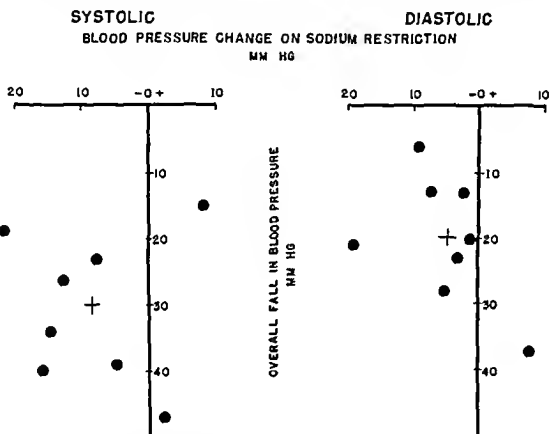
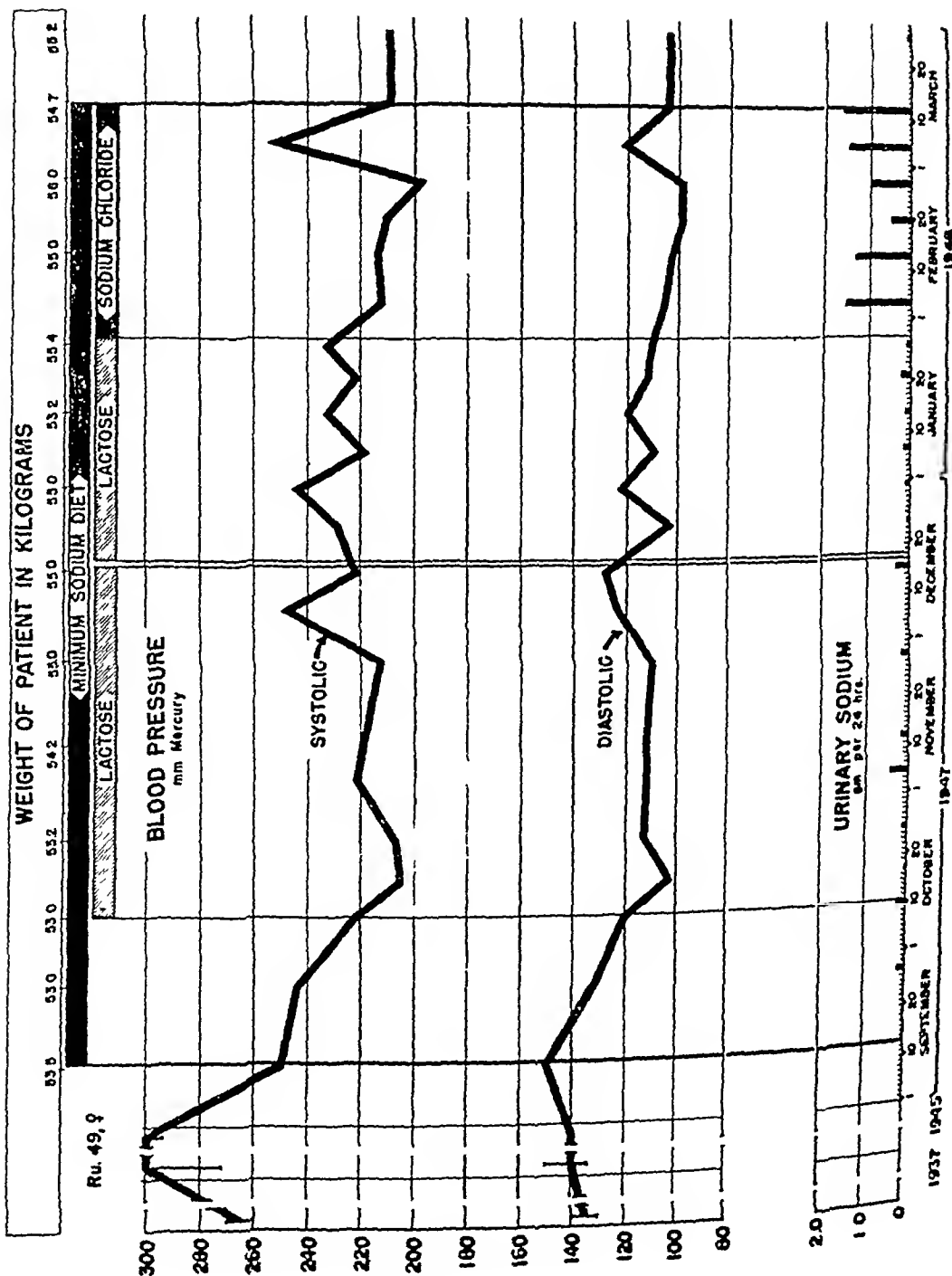


Fig 1—For the eight subjects in whom the effect of sodium restriction alone can be determined the blood pressure change ascribed to this restriction is compared with the maximum fall in pressure from the clinical records. Each dot represents one subject, the cross represents the average of all subjects. (All illustrations courtesy of the Veterans Administration, Washington, D. C.)

pressure, differences between observers, etc. If no analysis had been made of the change in blood pressure associated with sodium restriction alone, this entire effect could have been ascribed mistakenly to the therapeutic value of sodium restriction. In Fig 1, the fall in blood pressure ascribed to sodium restriction alone is compared with the maximum clinical fall in blood pressure based on the records. It will be noted that there is no correlation between the two sets of observations. Several individual records are presented in Figs 2 to 8. One patient whose blood pressure rose during the period of sodium restriction as compared with a period of liberal administration of sodium nevertheless had the greatest fall in blood pressure of any of the group on an overall basis (Fig 2). Moreover, this patient claimed great symptomatic improvement on this regimen. Her blood pressure was lowest during the six-week period in which the urinary sodium averaged 1.25 Gm per day (equivalent to 3.2 Gm of sodium chloride). The absence of correlation between the fall ascribable to sodium restriction alone and the general reduction in blood pressure that occurred during the course of the study



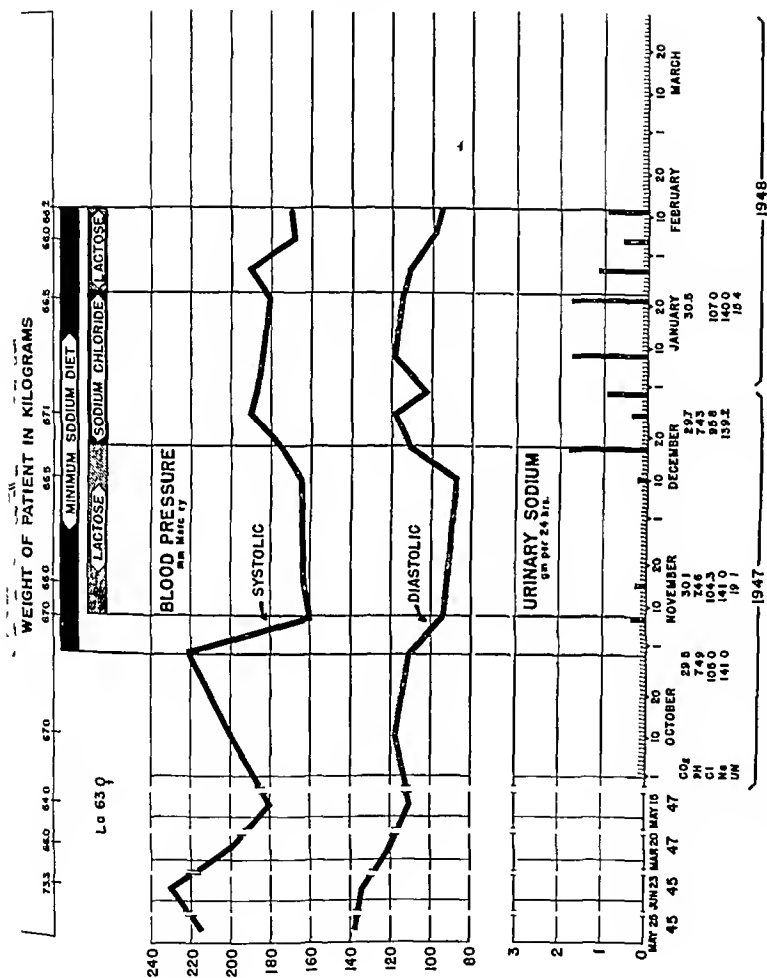
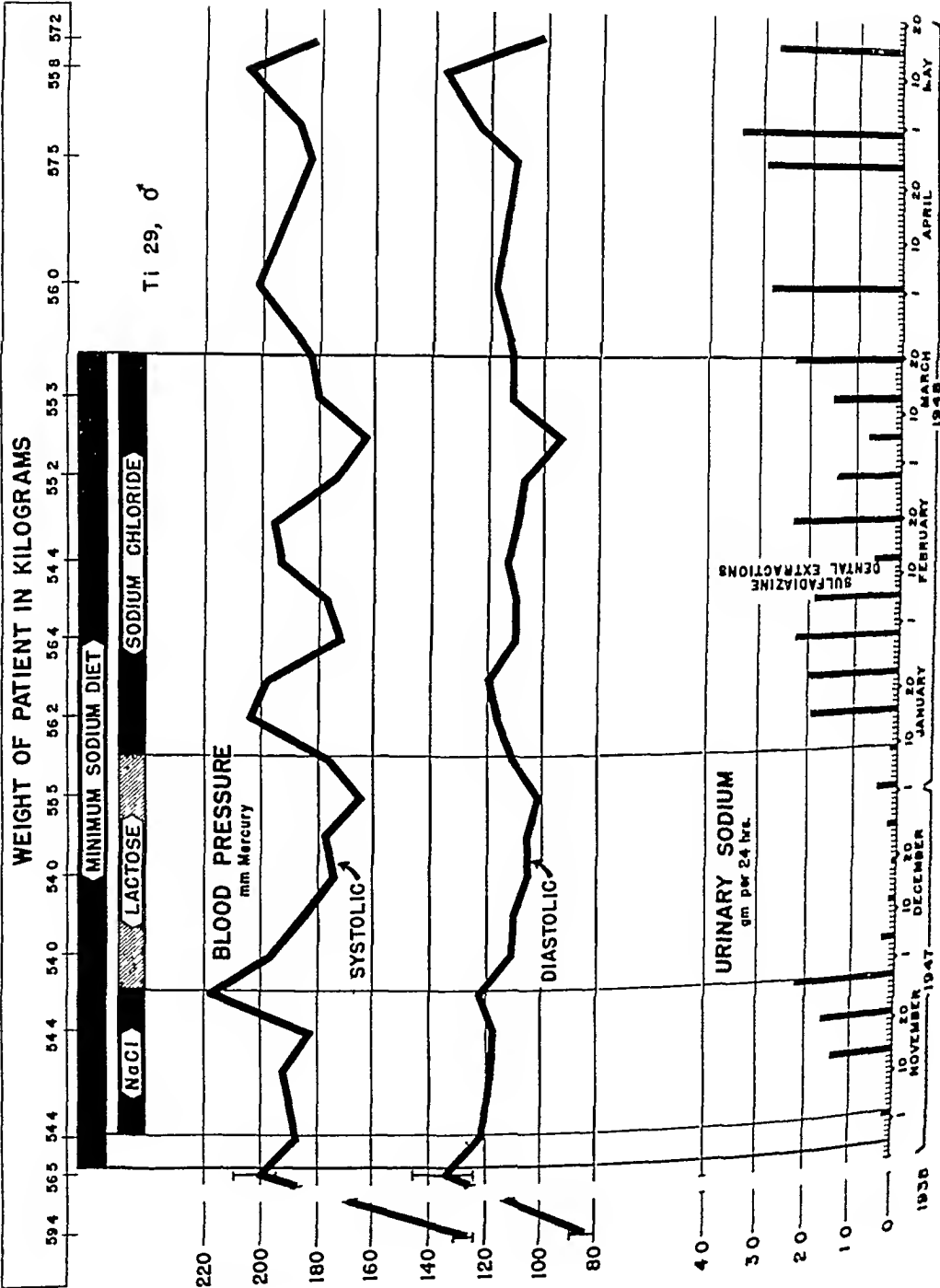
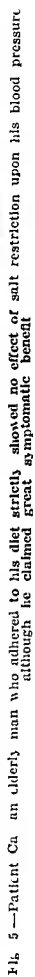
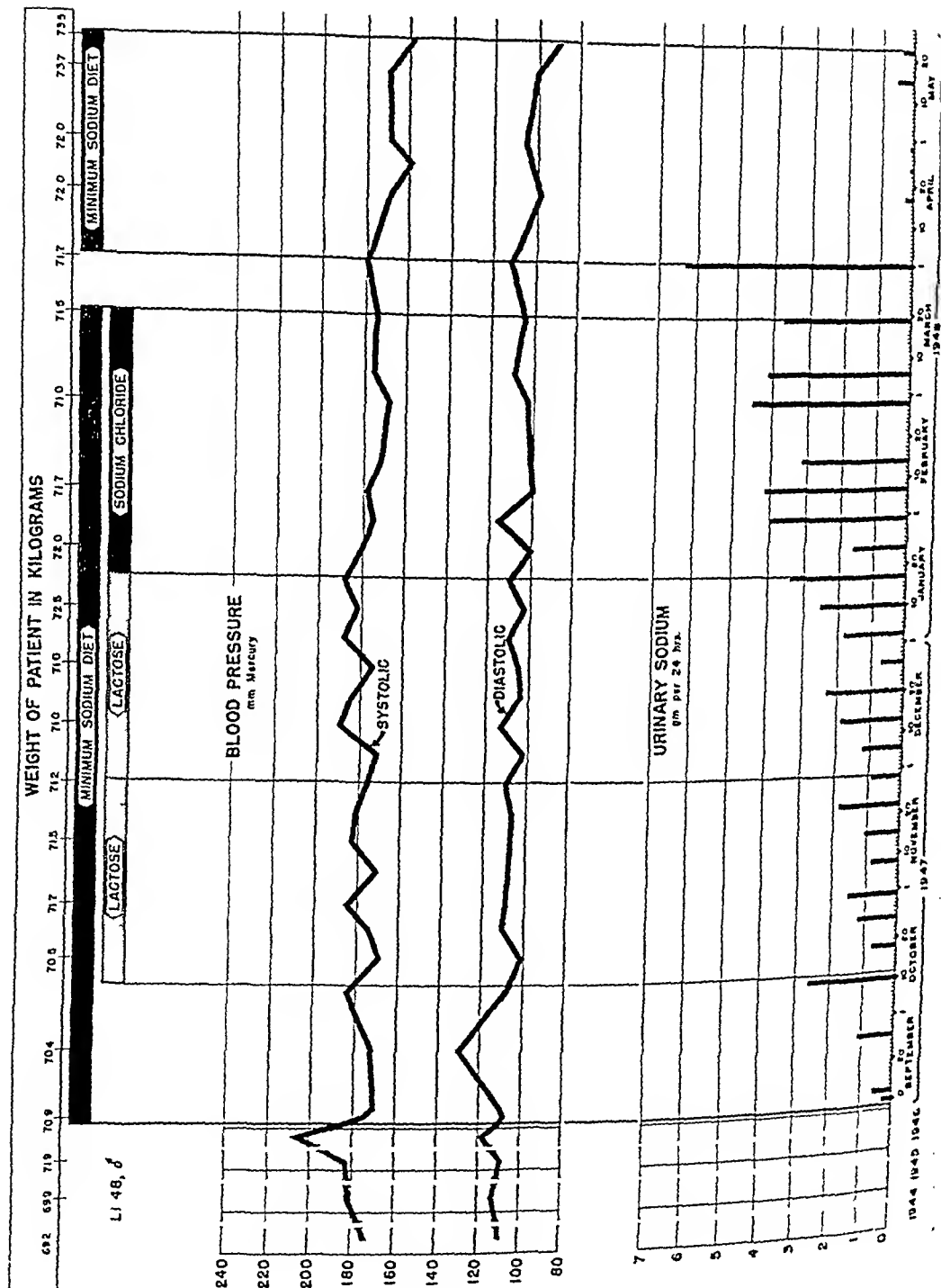


Fig. 3.—Patient La with previously recorded diastolic pressures over 110 showed a fall to normal levels during sodium restriction and a rise during supplementation with salt. Little weight change occurred.









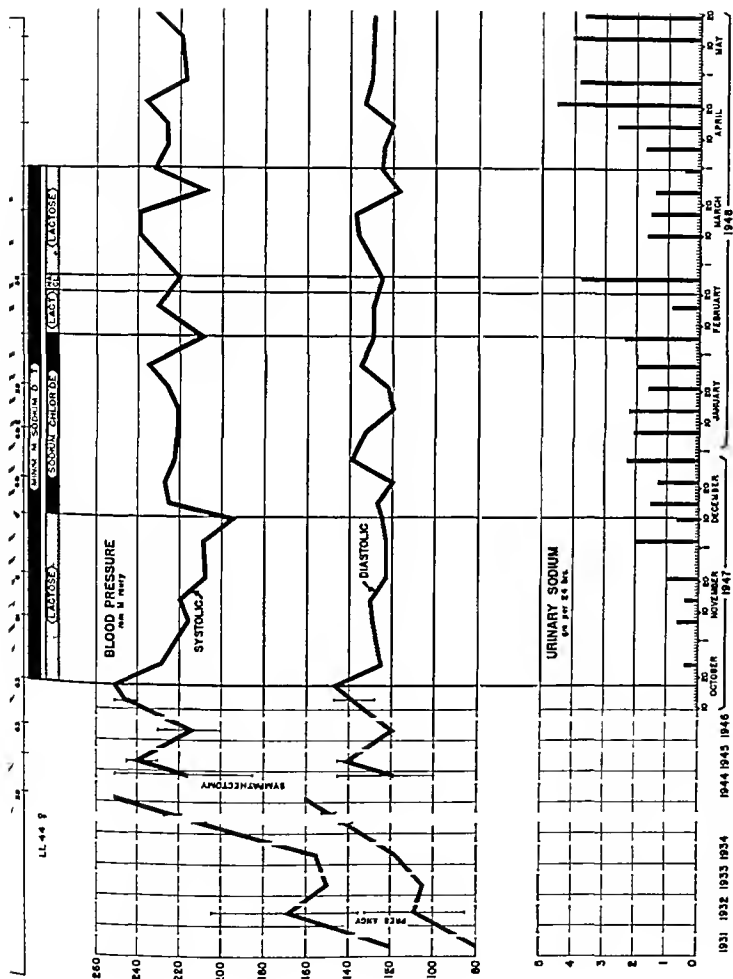
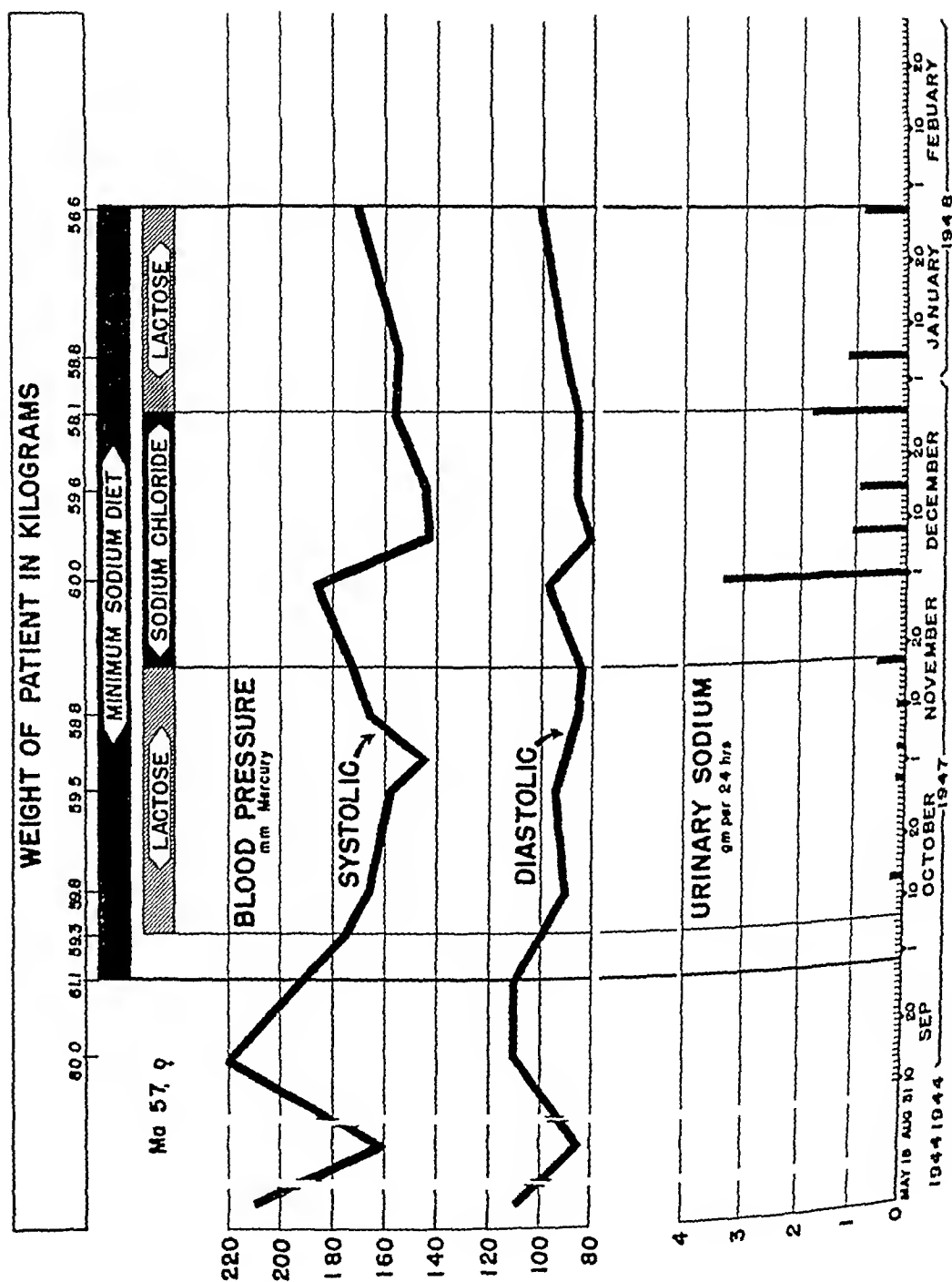


Fig. —Patient LL had been observed for sixteen years. The onset of her hypertension dated to pregnancy. Subsequent accretion was followed by a Smithwick sympathectomy in 1944. Her inability to remain on the diet invalidates the attempted evaluation of the effect of sodium restriction upon her blood pressure.



and the small number of cases of the experimental group make it rather pointless to present the individual cases in a search for the factors which make some patients susceptible to sodium restriction and others not. If the effect of this regimen is considered as the resultant of the two components, i.e. the effect of sodium restriction per se and the effect of all other factors, probably variation exists between individuals in each of these effects. It would be important to know how to select those patients who might have a good response to sodium restriction. It would appear of even greater importance to have criteria for selection of patients who respond to the factors other than sodium restriction. Both necessitate extension of our studies.

The foregoing comments deal only with the response of the blood pressure under the described regimen. A further factor of therapeutic importance arises when it is considered that the symptomatic effect of this regimen did not always parallel the change in blood pressure nor the variation in sodium intake. There were patients who felt better although the blood pressures did not fall and there were patients who felt better during a period when the sodium intake was higher than it had been before regardless of any change in blood pressure. Our brief studies do not permit analysis of these relationships nor do they control spontaneous variations in the level of the patient's symptoms.

We have been quite arbitrary in selecting the level of sodium excretion by which our patients have been grouped. Previous workers have maintained that a urinary sodium or chloride excretion equivalent to more than 0.5 Gm of sodium per day should not be considered an adequate degree of sodium restriction for the treatment of hypertension. It is quite possible that the blood pressure in some patients may fall with much more moderate sodium restriction than that which we have attempted. The average blood pressures for periods at different levels of sodium excretion reveal to some extent the individual and average response of patients when the degree of sodium restriction is varied. A more important objection to the conclusions of the study lies in the fact that the period of observation was arbitrarily limited to six weeks. In several instances it appeared that a change in blood pressure was becoming manifest toward the end of the six week period. This is in accord with the results of others who have pointed out that a delay in response may be noted. This is said to occur particularly during desalting. Such a phase displacement commented on previously will minimize any apparent effect of sodium restriction alone in this study. We do not feel that our data permit division of the experimental periods each into two periods of three weeks although this would be a way of approaching this valid criticism.

The study was designed to evaluate only one point and that was whether sodium restriction *alone* was capable of significantly reducing the blood pressure of hypertensive patients. Here 'significant' does not mean the significance to the patient or any therapeutic significance but simply whether sodium restriction had a *statistically significant* effect upon the blood pressure. This must be established before one can view in its proper light the result of the

complex alterations which ensue when a patient comes to the doctor's office or to the clinic and is advised to go on a rigidly restricted diet. It is important for the doctor to know whether the diet he advises is effective because of its sodium restriction, because of its specific composition, because of its psychological implication, or whether the patient appears to benefit because some active therapeutic effort is being made in his behalf or simply because the doctor is taking an interest in him. From the point of view of some patients (and physicians), the mechanism may not seem to be of importance, it often sufficing that the patient feels better and that the response satisfies his physician in whom he places his trust to keep him well. For the physician, this is self-deception.

There is great need for a wider realization of the profound physiologic, psychologic, psychiatric,<sup>20</sup> social, and economic alterations which may result from a diet such as this, apart from the biochemical and physiologic effects of sodium chloride depletion.

#### SUMMARY

An attempt was made to provide a maintenance diet with a sodium content restricted to less than 300 mg per twenty-four hours. This diet was offered to twenty-four ambulatory hypertensive patients on an experimental basis. In addition the subjects received either 4 Gm of sodium chloride or lactose in identical form over three periods of approximately six weeks each according to planned schedules. Neither the subjects nor the investigators were aware of the schedule being used and the subjects were not aware of the nature of or alteration in the medication. The criterion adopted for rigid restriction was the average excretion of less than 500 mg of urinary sodium in a weekly twenty-four hour sample. Blood pressures were recorded by a standardized technique approximately once each week. Eight subjects presented data for a period of restriction which could be compared with a period of greater sodium excretion. In these subjects the average diastolic blood pressure was 47 mm of mercury lower and the average systolic pressure 87 mm of mercury lower during the restricted period than during the period of sodium supplementation. Twenty-one subjects yielded data for at least two periods of a like order of sodium excretion from which the variances of the blood pressure for the group were determined. On this basis the probability that a difference as large as that experimentally observed would occur by random variation is less than one in one hundred for both the diastolic and the systolic values.

#### CONCLUSIONS

- 1 A diet rigidly restricted in sodium is difficult to administer successfully to ambulatory hypertensive subjects.
- 2 A difference of less than 5 mm of mercury in the average diastolic blood pressure was observed, which could be ascribed to the effect of the sodium chloride restriction alone.
- 3 This difference cannot reasonably be assigned to random variation.

4 Reduction in blood pressure observed when hypertensive patients are placed upon a diet which is restricted in sodium may be only partly due to sodium restriction. Some of the other factors which may be involved are pointed out but not analyzed in this study

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# INFLUENCE OF VARIOUS DISEASE STATES UPON THE FEBRILE RESPONSE TO INTRAVENOUS INJECTION OF TYPHOID BACTERIAL PYROGEN

WITH PARTICULAR REFERENCE TO MALARIA AND CIRRHOSIS OF THE LIVER

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CLINICAL experience during fever therapy has shown that patients given a series of injections of a bacterial pyrogen, such as that present in typhoid vaccine, develop a remarkable tolerance to the pyrogen, so that increasing doses must be given in order to produce comparable elevations in body temperature. This type of immune reaction or tolerance is of short duration and is apparently not dependent on the presence of specific antibodies<sup>1</sup>. Experiments in rabbits indicate that it is related to an increased ability of the reticulo-endothelial system to remove the pyrogen from the circulation<sup>2</sup>. The question arises whether a similar tolerance develops in human beings during the course of certain febrile diseases. If such alterations do occur, patients convalescent from these diseases would be expected to show very little febrile reaction to intravenous injection of a bacterial pyrogen. One observation pertinent to this problem was reported by Howard<sup>3</sup> who, in studying the effect of fever on protein metabolism, found that he was unable to elicit an appreciable febrile response to typhoid vaccine in a patient who had recently recovered from malaria. Morgan<sup>4</sup> reported that patients convalescent from typhoid and paratyphoid fever showed no significant febrile reactions to intravenous injection of purified extracts of *Escherichia typhosa* and *Shigella dysenteriae*. This material, however, produced marked febrile reactions in normal individuals. The present report summarizes observations on the febrile response of patients with various diseases to intravenous injection of a small dose of typhoid vaccine.

## METHOD

The vaccine employed was a suspension of heat killed *E. typhosa* containing approximately one billion organisms per milliliter. The standard dose was 0.5 ml injected intravenously. Previous experience with this vaccine had shown that this dose produced little symptomatic discomfort other than a sensation of chilliness followed by a rise in temperature of 1.5° F to 3° F with temperature returning to normal in four to six hours. Temperature measurements were made with a constant recording rectal thermometer. Readings were begun one hour previous to the injection and continued at thirty minute intervals for eight hours afterward. The febrile response was plotted on graph paper and the temperature previous to injection was taken as the base line. The area between the base line and the course of the febrile response was measured with a planimeter. The size of it, when expressed in square units of the planimeter, was called the "fever index".

The subjects studied can be divided into five main groups:

1. A 'normal' group of sixteen febrile patients, receiving penicillin treatment for asymptomatic neurosyphilis.

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2 Twenty two patients convalescent from a variety of acute infectious diseases, including seven with pneumonia four with gonorrheal arthritis three with typhus fever three with tularemia, two with typhoid fever two with meningitis and one with rheumatic fever. The vaccine was administered to these patients two or three days after they had recovered from their illnesses and had become afebrile.

3 Twenty patients who had just completed a course of malarial fever therapy for neurosyphilis. The test was carried out on the second or third day following the last malarial paroxysm.

4 Fourteen patients with cirrhosis of the liver.

5 Thirteen patients with jaundice ten of whom had viral hepatitis and three of whom were thought to have biliary obstruction. All of the patients with liver disease were tested at a time when their temperatures had been normal for two or three days.

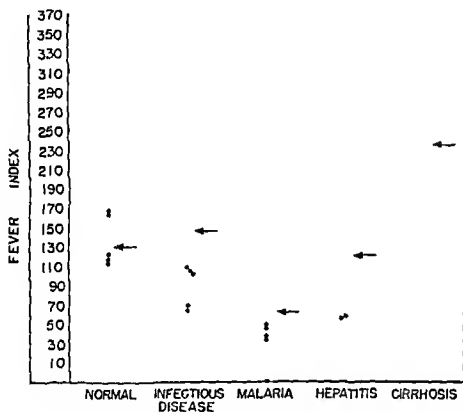


Fig. 1.—The febrile response to typhoid vaccine in various disease states. The arrow indicates the mean response in each group of patients.

## RESULTS

The febrile responses following injection of typhoid vaccine in the different groups are shown in Fig. 1. It will be observed that in the "normal" subjects the range of the fever index varied from 43 to 262 the mean being 132. This quantitative expression of both the height and the duration of fever indicates a rise of  $1.5^{\circ}\text{C}$  to  $3^{\circ}\text{C}$  the temperature returning to normal usually within six hours.

In patients recently convalescent from acute infections the febrile responses were comparable to those of the "normal" subjects the mean value of the fever index in this group being 148. The four highest values were in two patients with typhoid fever and two with tularemia. The two patients with typhoid fever the only ones in this study with this condition exhibited

an increased sensitivity to pyrogen, a finding at variance with that of Morgan<sup>7</sup> who reported a decreased febrile response in a larger number of patients convalescing from this illness. One other patient with tularemia showed a febrile response similar to that appearing in the normal subjects.

The twenty patients who had recently recovered from malaria reacted to the typhoid vaccine with less fever than the preceding groups, their mean fever index being only 63. This finding is similar to that noted by Howard in one case. Comparable observations were made by Habetm,<sup>9</sup> who found that the injection of pyrogenic nuclein, made from yeast, failed to produce any febrile response in a few patients with malaria.

Patients with cirrhosis of the liver showed greater than normal response, the fever indices ranging from 140 to 352, the mean value being 236.

In the group of thirteen patients with jaundice, twelve showed responses within the "normal" range, whereas one with infectious hepatitis had an exaggerated febrile response. This person had been ill for approximately four months, and liver function tests showed considerable evidence of hepatic insufficiency at the time the test was performed.

#### DISCUSSION

The increased sensitivity to pyrogen observed in patients with chronic liver disease may possibly be caused by impaired reticuloendothelial function in the liver and spleen, or may be related to some metabolic disturbance. Patients with cirrhosis have been observed to develop fever following the intravenous administration of salt-poor albumin.<sup>7</sup> There is also a definite impression that certain preparations of albumin produced febrile reactions in persons with cirrhosis, but not in patients with hypoalbuminemia due to other causes.<sup>8</sup> The patients in the present study with acute hepatitis or obstructive jaundice, however, did not exhibit abnormal responses to typhoid vaccine. Experiments with rabbits in this laboratory have also shown that acute liver damage, produced by chloroform, did not lead to abnormal febrile response to typhoid pyrogen.

The diminished response to the pyrogen in patients with malaria may possibly be associated with the great proliferation in reticuloendothelial elements known to occur in this infection. The stimulation of the reticuloendothelial system produced by malarial fever and typhoid vaccine fever therapy has been offered as an explanation for their curative effect in neurosyphilis.

The patients in this study with febrile diseases other than malaria developed little if any tolerance to bacterial pyrogen. Morgan,<sup>7</sup> however, has observed that patients convalescent from typhoid and paratyphoid fevers showed tolerance to injection of purified extracts of typhoid and dysentery bacilli, whereas the reaction observed in five patients recovering from other febrile diseases was comparable to that of normal subjects. Bennett found that rabbits surviving pneumococcal and colon bacillus infection did not show a diminution in febrile response to typhoid or colon bacillus pyrogens.<sup>9</sup>



## SUMMARY

The febrile response to typhoid bacterial pyrogen was observed in a group of eighty five patients with various diseases. The mean response elicited by patients with a variety of infections, such as pneumonia, hepatitis, typhus fever, and tularemia, was the same as that observed in normal individuals. Patients with malarial fever, however, appeared to be comparatively resistant to the pyrogen. Patients with cirrhosis of the liver exhibited an exaggerated response.

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# THE IN VIVO ACTION OF AUREOMYCIN ON PLEUROPNEUMONIA LIKE ORGANISMS ASSOCIATED WITH VARIOUS RHEUMATIC DISEASES

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THE efficacy of gold compounds in animals experimentally infected with animal strains of pleuropneumonia-like organisms has been demonstrated by various observers<sup>1-6</sup>. A strain of *Streptobacillus moniliformis* recovered from the joint fluid of a patient produced arthritis in mice with great regularity. Arthritis could be prevented by the simultaneous administration of gold and the infecting agent, but arthritis once established was unaffected by the use of gold. The transformation of this strain of *S. moniliformis* into the pleuropneumonia-like form was readily demonstrated.

A patient with nonspecific urethritis and prostatitis from whom pleuropneumonia-like organisms were recovered has been treated with Myochrysin without improvement<sup>8</sup>. Sulfonamides and penicillin have been found ineffective both in vitro and in vivo in animal and human infections with these organisms<sup>7-9</sup>. Streptomycin has been observed to have an effect on L organisms and has produced a rapid disappearance of symptoms in patients with an associated infection due to these organisms<sup>8-10</sup>. However, pleuropneumonia like organisms have been observed to persist in a case of nonspecific urethritis following streptomycin therapy<sup>8</sup>. It has been pointed out repeatedly that L organisms may be of significance in human joint diseases but a definite correlation has not yet been established<sup>8-11-12</sup>.

## OBSERVATIONS

In the present study, patients with rheumatoid arthritis, from whom cultures of prostatic secretion yielded pleuropneumonia like organisms, were observed for one year or more. An effort was made to determine whether or not under prolonged gold therapy for the rheumatoid arthritis the organisms could be eliminated in vivo in human beings. In all patients of this group, L organisms could be recovered with great regularity in spite of continued gold therapy and satisfactory clinical remissions.

In one of these patients, each of fourteen successive prostatic cultures obtained over a period of eighteen months was positive for L organisms. After thirteen months of gold therapy (10 mg the first week, 25 mg the second week, 50 mg weekly for nineteen weeks, 50 mg monthly for eight months, total dosage, 1,385 mg), myochrysin was discontinued because of toxicity. Three

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mouths after cessation of therapy the rheumatoid arthritis relapsed and aureomycin\* therapy was instituted. There was a complete disappearance of pleuropneumonia like organisms on six successive culture attempts during and following aureomycin therapy.

Disappearance of L organisms from the genital tract under aureomycin therapy has been demonstrated in a total of nine patients (three men, six women: two cases of erythema nodosum, one of rheumatic fever, five of rheumatoid arthritis, one of nonspecific urethritis). In two of the female patients L organisms reappeared after discontinuation of the drug, indicating either insufficient dosage or bacteriostatic rather than bactericidal activity of aureomycin. One of these patients was re-treated with aureomycin and again prompt elimination of the organisms was achieved.

Four patients with nonspecific urethritis have been treated with aureomycin. Two of these patients had joint and muscle complaints in addition to the urethritis. In only one patient cultures of prostatic secretion yielded abundant growth of pleuropneumonia like organisms. There was prompt response to aureomycin (20 Gm per day for four days) confirming the observation of Collins and co-workers.<sup>12</sup> Complete relief of symptoms occurred after forty-eight hours of therapy. Cultures taken thirty-six hours and one ten and eleven weeks after institution of aureomycin therapy were negative for pleuropneumonia like organisms. In spite of negative cultures the patient had a recurrence of burning on urination but no return of urethral discharge. The burning sensation has persisted in the absence of further treatment. This observation, a recurrent clinical symptom and negative cultures in a patient in whom L organisms were demonstrated previously, supports the impression that absence of demonstrable L organisms does not rule out the possibility that disease was initiated by infection with them. The inability to demonstrate pleuropneumonia like organisms may be due to the inadequacy of the present culture methods. This failure to culture organisms may also parallel the findings in *S. mouliformis* infections in which in the later stages of the disease it may be impossible to recover the organism although clinical activity persists. These speculations would explain the frequently noted lack of correlation between clinical and laboratory findings.

In three of the four patients with nonspecific urethritis pleuropneumonia like organisms could not be demonstrated in repeated cultures prior to aureomycin therapy. One patient was treated for an attack of three months' duration which had not improved under penicillin therapy. One gram of aureomycin was given daily for seven days. The patient was asymptomatic on the last day of treatment. Burning on urination recurred two weeks after cessation of therapy and four weeks after discontinuation of aureomycin morning discharge was again present. A second course of aureomycin was given consisting of 1 Gm daily for five days and 20 Gm daily for three weeks. The discharge ceased after five days of therapy, burning diminished during treatment and cleared completely a few days after cessation of therapy. The patient has remained asymptomatic during a five week follow up period. A second patient

\*We are indebted to the Lederle Laboratories for part of the aureomycin used in this study.

became asymptomatic following forty-eight hours of treatment (10 Gm per day for seven days), but mild burning on urination developed one week after aureomycin had been discontinued. This patient has not been re-treated. The third patient presented a two-year history of urethritis. Burning and urethral discharge were present at the onset of the disease. After three months of the illness the discharge cleared spontaneously, but burning and frequency of urination persisted with the gradual development of arthralgia, myalgia, and fatigue. Aureomycin therapy (0.5 Gm per day for four weeks, 1.0 Gm per day for ten days) appeared ineffective. However, when the dose of aureomycin was increased to 2.0 Gm per day striking improvement was noted. After the patient had been on this larger dosage for three weeks, he noted for the first time in two years absence of burning on urination and was completely free of myalgia, joint pain, and fatigue. Treatment was continued for a total of ten weeks. There was no recurrence of symptoms. There has been inadequate time for follow-up studies.

Twenty-five patients representing a variety of rheumatic diseases (rheumatoid arthritis, Marie-Strumpell spondylitis, rheumatic fever, erythema nodosum) have been treated with aureomycin (Tables I and II).<sup>14</sup> Cultures from the cervix and prostatic secretions yielded pneumopneumonia like organisms in eight of these patients. Cultures were negative for L organisms in seventeen patients.

Subjective and objective improvement was observed in seventeen of these twenty-five patients. Two (Patients R. H. and E. V.) were symptomatically improved. One case of rheumatic fever (Patient J. T.) could not be evaluated because salicylates were given simultaneously. Two patients with Marie-Strumpell spondylitis (Patients D. T. and L. A.) showed no demonstrable improvement after five and eight weeks of treatment. One individual with Marie-Strumpell spondylitis and peripheral joint involvement (Patient J. B.) experienced temporary marked symptomatic improvement followed by an acute

TABLE II EFFECT OF AUREOMYCIN THERAPY ON RHEUMATIC FEVER AND ERYTHEMA NODOSUM

DIAGNOSIS	PATIENT	SEX	DEGREE OF INVOLVEMENT	DURATION OF ILLNESS	AUREOMYCIN		THERAPEUTIC RESPONSE	ORGANISMS	
					GM / DAY	DURATION (DAYS)		BEFORE THERAPY	AFTER THERAPY
Rheumatic fever	J. T.	F	Chronic and acute, severe	14 yr	2.0	17	?	+	0
	S. P.	F	Chronic and acute, severe	7 yr	0.75	45	Good	-	
	P. W.	M	Acute, severe	7 yr	2.0	10	Good	0	
Erythema nodosum with fever and joint involvement	C. M.	F	Acute, severe	2 mo	2.0 1.0	5 11	Good	+	0
	N. C.	F	Acute, severe	2 mo	0.5 1.0	5 20	Good	+	0

Minus sign denotes that the determination was not made

exacerbation of the disease. Two persons (Patients F W and J A) with rheumatoid arthritis did not improve. It may be of significance (Table I) that the aureomycin blood serum levels were uniformly low in those patients in whom little or no clinical improvement was noted.

The relation between dosage of aureomycin, aureomycin blood serum levels, temporary or permanent elimination of *L* organisms and clinical response will require further study. Disappearance of *L* organisms in the patients with positive cultures was more rapid than the clinical improvement noted in any single case with the dosage of aureomycin and the route of administration employed. In one case of erythema nodosum as little as 0.25 Gm of aureomycin daily was all that was necessary to eliminate pleuropneumonia like organisms from the cervix. In this case 10 Gm per day of the antibiotic was required to produce a favorable clinical response. One patient in whom *L* organisms disappeared promptly failed to show clinical response. The patients with negative cultures for *L* organisms responded as well as those from whom the organisms could be recovered.

There was an initial exacerbation of the illness in a number of cases of rheumatoid arthritis and Marie Strimpell spondylitis comparable with that frequently observed by us in rheumatoid arthritis treated with gold. This exacerbation of muscle and joint symptoms varied in intensity and duration, but uniformly disappeared under continued therapy. Two patients who had fever prior to therapy developed an initial transient additional rise in temperature on 20 Gm of aureomycin daily. The three other febrile patients in the rheumatic fever erythema nodosum group in whom aureomycin did not exceed 10 Gm per day did not have this additional febrile response.

It was observed early in the course of this study that some patients who failed to improve on 1 Gm of aureomycin daily responded favorably when the dosage was increased to 20 Gm per day. Therefore the 2 Gm dose was selected whenever the patient could tolerate this amount. In most patients improvement was noted one to four weeks after initiation of therapy and continued in a manner comparable to that observed with successful gold therapy of rheumatoid arthritis. The high incidence of favorable response to aureomycin suggested an effect which was more than a coincidental finding. Aureomycin therapy may not be suitable in cases of rheumatic fever because of the initial exacerbation of symptoms noted. Smaller initial doses than those employed in the present study may eliminate this exacerbation.

Kuzell<sup>1</sup> has reported that the effect of aureomycin was similar to that of gold in the control of rat arthritis experimentally produced with the *L*<sub>4</sub> strain. Brown<sup>16</sup> has noted the effect of aureomycin in human beings with rheumatic diseases. It would appear that aureomycin and undoubtedly other antibiotics with a similar effect on *L* organisms may assume an essential role in the understanding of the relationship of pleuropneumonia like organisms to rheumatic diseases. Preliminary studies indicate that the effect of chloromycetin may be comparable to that of aureomycin. In one female patient with severe progressive rheumatoid arthritis repeated cervical cultures were done before and

after administration of 10 Gm of chlormycetin daily. Prompt elimination of L organisms was achieved. There was an exacerbation of symptoms followed by rapid major clinical improvement within three weeks.

#### SUMMARY

1 Oral administration of aureomycin has produced uniform disappearance of demonstrable pleuropneumonia-like organisms from the genitourinary tract of individuals with and without joint disease. L organisms were not eliminated by gold therapy.

2 Elimination of L organisms by aureomycin or by other antibiotics producing a similar effect may be of importance in the understanding of the possible relationship of pleuropneumonia-like organisms to articular diseases.

3 The effect of aureomycin therapy in twenty-five cases of rheumatic disease has been reported.

4 The ultimate value of aureomycin in the treatment of rheumatic diseases must await prolonged clinical trial and follow-up study.

We are indebted to Dr. W. D. Jarman for permitting the use of his case in this study (one case of nonspecific urethritis).

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# SONIC VIBRATION LEPTOSPIRAE AS ANTIGENS IN THE COMPLEMENT FIXATION TEST FOR THE DIAGNOSIS OF LEPTOSPIROSIS

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IT IS recognized that in the majority of cases of human and animal leptospirosis a diagnosis cannot be made without recourse to one or more laboratory procedures

Leptospirae can be demonstrated by dark field microscopic examination of the blood during the septiceemic stage of the disease which usually lasts for seven days. Also at this time inoculation of young hamsters with whole blood from patients infected with *Leptospira icterohaemorrhagiae* and *Lept. canicola* generally results in a fatal leptospirosis in the test animal<sup>1</sup>. The leptospirae disappear from the patient's blood stream during the second week and then appear in the urine. Agglutinating lysins and complement fixing antibodies usually begin to appear in the patient's serum toward the end of the first week and are present in readily measurable amounts about the twelfth to fourteenth day after the onset of illness. The maximum titer is reached within three to four weeks and in most instances antibodies are detectable for several months to years.

The tests most frequently used for the demonstration of antibodies in the patient's serum are the macroscopic<sup>2</sup> and microscopic agglutination<sup>3</sup> and agglutination lysis tests<sup>4</sup>. These procedures require personnel properly trained in the techniques and necessitate the maintenance of suitable cultures of leptospirae. For this reason their use is limited to only a few laboratories.

Because a simple and reliable diagnostic test for leptospirosis was not available a study was undertaken to develop an antigen for use in a complement fixation test that could be performed in any laboratory conducting the Wassermann or similar complement fixation tests. In the past, the complement fixation test for the diagnosis of leptospirosis has received little attention apparently because of the difficulty of preparing suitable antigens. Pot and Dorniel<sup>5</sup> and Boerner and Lukens<sup>6</sup> have reported on two different methods of preparing antigens for use in the complement fixation test for the diagnosis of Weil's disease. However, Boigen<sup>7</sup> concludes that the lack of agreement between the agglutination reactions and the complement fixation test is due to sources of error in technique or to unstable or inefficient antigens. While we utilized antigens prepared by present available methods our results also have been unsatisfactory. Therefore experiments were undertaken to produce a suitable antigen and it was found that leptospirae ruptured by sonic vibration yielded an antigen of considerable specificity and sensitivity.

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## MATERIALS AND METHODS

*Preparation of Antigen*—Although there are sixteen serologically recognized species of leptospira, only two, *Lept icterohaemorrhagiae* and *Lept canicola*, are known to infect human beings and dogs in the United States, therefore, antigens were prepared from these two species. Antigens were prepared from strains of *Lept icterohaemorrhagiae* and *Lept canicola* isolated in this laboratory from young hamsters inoculated with whole blood from naturally infected dogs.<sup>1</sup> Erlenmeyer flasks of 250 ml capacity containing 50 ml amounts of Stuart's medium<sup>8</sup> were inoculated with 50 ml of a 3 day old culture of leptospira and incubated at 29 to 30° C for six days. At the end of this period 0.05 ml of formalin was added to each flask and the cultures were held at 29 to 30° C for an additional twenty-four hours. The killed cultures were transferred in 50 ml amounts to plastic tubes and centrifuged at 17,000 r.p.m. for fifteen minutes in an International Model PR 1 refrigerated centrifuge maintained at 0 to 3° C. The supernatant fluid was decanted and the packed leptospirae were collected and washed three times with 0.85 per cent saline solution buffered at pH 7.0. The washed organisms were then resuspended in buffered saline solution to one tenth of the original volume of the culture medium. Twenty milliliter aliquots of the concentrated suspension of the leptospirae were exposed to sonic vibration at 9,400 cycles per second for ten minutes in a water cooled Rytison Type R 22 magnetostriiction oscillator maintained at 100 volts. The material was then pooled and Merthiolate added to 1:1,000 final concentration to constitute the species specific stock antigen. Antigens refrigerated at 4° C have remained stable and specific for at least six months.

*Complement Fixation Tests*—The complement fixation technique used at the Army Medical Department Research and Graduate School is employed.

*Complement Titrations*—The 1:30 dilution of complement is titrated to determine the unit and two full units contained in a 0.5 ml volume are used for the test. (For example, if hemolysis is just complete in the tube containing 0.14 ml of complement, then 0.16 ml is taken as the unit.)

*Antigen Titrations*—Titrations have shown the optimum dilution of the stock antigen to be 1:15, with the unit ranging from 0.02 to 0.05 ml of this dilution. Three exact units of antigen contained in a 0.25 ml volume are employed.

*Inactivation of Sera*—Human sera are inactivated at 56° C for twenty minutes, dog and rabbit sera at 62 to 63° C for forty minutes in a dilution of 1:1. In the routine test, 0.25 ml of twofold dilutions, from 1:1 through 1:32 and in a quantitative test 1:1 through 1:1,280, of the inactivated serum is used.

*The Test*—To 0.25 ml amounts of the sera dilutions three units of antigen and two full units of complement are added. The tubes are refrigerated at 4 to 6° C for sixteen to eighteen hours. On removal, the tubes are left at room temperature for fifteen to twenty minutes and 0.5 ml of sensitized cells consisting of 0.25 ml of a 3 per cent suspension of sheep cells and 0.25 ml of amboceptor containing three units is added. The tubes are incubated at 38° C for thirty minutes in the water bath and the test is read.

*Controls*—The usual hemolytic and positive and negative control sera are set up.

## RESULTS

Parallel complement fixation and microscopic agglutination tests were made on three groups of human sera. Group I consisted of twenty-five random serum specimens submitted to this laboratory from a wide variety of disease processes other than proved cases of Weil's disease. Group II included ten sera from syphilitic patients giving positive Wassermann reactions. Group III included sera from twenty-one proved cases of Weil's disease. In none of the random specimens in Group I were either complement-fixing or agglutinating antibodies against either leptospira antigen detected.



All of the ten positive Wassermann sera of Group II likewise failed to react with leptospira antigens in either type of test

On the other hand, definite antibody responses were elicited in all of the sera of patients diagnosed as having Weil's disease (Group III). Two examples are shown in Table I

TABLE I. THE OCCURRENCE OF COMPLEMENT FIXATION AND AGGLUTINATION ANTIBODY IN TWO CASES OF HUMAN LEPTOSPIROSIS

SERUM FROM CASE	DAYS SINCE ONSET OF DISEASE	TITER OF COMPLEMENT FIXATION TEST		TITER OF MICROSCOPIC AGGLUTINATION TEST	
		LEPT CANICOLA ANTIGEN	LEPT ICTEROHAEMORRHAGIC ANTIGEN	LEPT CANICOLA ANTIGEN	LEPT ICTEROHAEMORRHAGIC ANTIGEN
1	5	1 8	1 8	0	0
1	28	1 128	1 128	1 10 000	1 1 000
2	6	1 16	1 16	0	0
2	17	1 32	1 32	1 10	1 300
2	42	1 64	1 128	1 100	1 1 000

Case 1 was a patient whose first symptoms of illness were general malaise, headache, conjunctival injection and pyrexia. Five days after onset, the serum showed a complement fixation titer of 1 8 against both *Lept canicola* and *Lept icterohaemorrhagiae* antigens. Another specimen of serum obtained on the twenty eighth day of illness showed a rise in complement fixation titer to 1 128 against both antigens. The microscopic agglutination test on the first specimen was negative while the second specimen reacted with *Lept canicola* and *Lept icterohaemorrhagiae* antigens at 1 10 000 and 1 1 000 dilutions respectively.

Case 2 was that of an attendant in a small animal hospital who developed general malaise, chills and fever. His serum fixed complement with both leptospira antigens at a dilution of 1 16 on the sixth day of reported illness. By the seventeenth day the titer against both antigens had risen to 1 64 and on the forty second day the complement fixation titer against *Lept canicola* was 1 64 and that against *Lept icterohaemorrhagiae* 1 128. Parallel studies with the microscopic agglutination technique showed negative results on the first specimen of serum while the second reacted with *Lept canicola* at 1 10 and *Lept icterohaemorrhagiae* at 1 300. The third serum specimen had a positive agglutination titer of 1 100 for *Lept canicola* and 1 1 000 for *Lept icterohaemorrhagiae*. These observations suggest that antibody formation in leptospirosis may be detectable by the complement fixation test before it is demonstrable by the microscopic agglutination test.

Nineteen of the sera in Group III from human cases of leptospirosis occurring in the Far East were obtained through the courtesy of Dr. Pierre Leprieux of the Pasteur Institute. These cases had been diagnosed at point of origin by use of the agglutination lysis test. Unfortunately serial specimens were not obtained for comparative studies but the positive findings obtained on single specimens as shown in Table II were in accord with the comparative results of the complement fixation and microscopic agglutination tests noted in Table I.

# EXPERIMENTAL VASCULAR DISEASES DUE TO DESOXYCORTICOSTERONE ACETATE AND ANTERIOR PITUITARY EXTRACT

## I COMPARISON OF FUNCTIONAL CHANGES

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**I**NJECTIONS of crude anterior pituitary preparations elicit in rats morphologic and physiologic changes similar to those caused by desoxycorticosterone acetate (Selye, 1944). Hence Selye (1944) has suggested that the pathogenesis of hypertension, nephrosclerosis, periarteritis nodosa, and certain myocardial lesions can best be explained on the basis of pituitary adrenal activities. The postulated chain of events would be stress  $\rightarrow$  anterior pituitary (ACTH)  $\rightarrow$  adrenal cortex (desoxycorticosterone like compounds)  $\rightarrow$  kidney, blood vessels, heart. The posited identification of the effects of anterior pituitary preparations with those of desoxycorticosterone acetate is lacking in detail. Thus hypertension was estimated either from heart weight (Selye and Stone, 1946) or from single direct measurements of carotid arterial pressure (Dontigny, Hay, Prado, and Selye, 1948).

As part of a more definitive comparison, the present report embodies a survey of the functional effects of anterior pituitary preparations and desoxycorticosterone acetate accomplished by serial measurements of arterial pressure and water diuresis in groups of animals treated with these substances singly or in combination. Diuresis as well as blood pressure was a major criterion in the comparison because of the well-known diuretic effect of desoxycorticosterone. Organ weights also were measured and compared.

## I EFFECTS OF CRUDE ANTERIOR PITUITARY PREPARATION AND DESOXYCORTICOSTERONE ACETATE

### *Experimental—*

Sprague Dawley female albino rats were fed a high protein diet (75 per cent Purina fox chow plus 25 per cent casein) and given 1 per cent saline solution as drinking water. Further "sensitization" (Selye, Stone, Nielsen, and Leblond, 1945) to the hormones was effected by unilateral nephrectomy. Rats were divided into four groups. Group I served as control, Group II received 25 mg of desoxycorticosterone acetate (DOCA) a day, Group III, 30 mg of a suspension of anterior pituitary powder (APP), Group IV, a combined treatment of DOCA and APP at the same dosage levels as in Groups II and III respectively. Group IV was added in order to see whether the two hormonal preparations would act additively or synergistically.

The steroid was suspended in water in a concentration of 10 mg per cubic centimeter. The anterior pituitary powder, prepared by lyophilization of cattle glands, was suspended in a concentration of 50 mg per cubic centimeter in 1 per cent saline containing 10 per cent of

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alcohol and filtered through gauze. The solution was kept in the icebox and made up weekly. Injections were begun immediately after unilateral nephrectomy. The animals were placed in metabolism cages in order to measure diuresis.

Blood pressure was measured by the plethysmographic method of Williams, Harrison, and Grollman (1939) as modified by Kempf and Page (1942). Each value recorded is an average of two to three close consecutive readings. Reproducible results are obtained in nonanesthetized rats under the following conditions: (1) Animals are kept constantly at a temperature of about 30° C. (2) at the time of the determination each rat is placed in a constant temperature (40° C) box for a period of three minutes and (3) the tail is

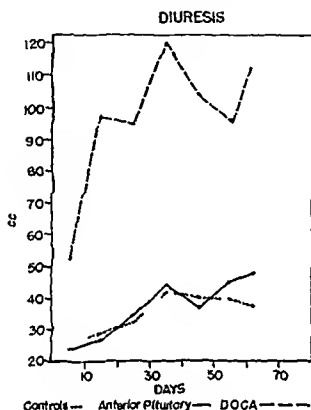


Fig. 1—Effect of desoxycorticosterone acetate and anterior pituitary preparations on urine formation

compressed and emptied of blood in the plethysmograph just prior to increasing pressure in the air cuff. From repeated observations on 132 rats blood pressure was found to vary from 70 to 130 mm Hg (average 99) with 68 per cent of the animals in the 90 to 110 mm Hg range. Some of these measurements were compared with those of an electrically recording plethysmograph (Olmsted, Corcoran, Glaser and Page, 1948) from time to time as a further check.

Unless otherwise stated animals were sacrificed on the sixty first day and autopsied. Heart, kidney, ovaries, and adrenals were weighed and sectioned for histologic studies after fixation in Zenker's solution. These detailed histologic observations will be the subject of another paper.

### Results —

Observations summarized in Figs. 1 and 2 and in Table I are averages of two series of experiments in which the mean initial body weights of the rats were respectively 103 and 219 grams. Results were the same in the two series, so that they are reported jointly. Weights of heart and adrenals are expressed in milligrams per 100 grams of body weight and kidney weights in milligrams per 100 sq cm of body surface. The body surface was calculated according to the formula:  $SA = Wt \times 0.66 \times 11.23$

TABLE I EFFECTS OF DESOXYCORTICOSTERONE ACETATE (DOCA) AND ANTERIOR PITUITARY PREPARATION (APP) ALONE OR IN COMBINATION

GROUP	NUMBER OF RATS	MEAN BODY WEIGHT CHANGES	HEART (MG /100 GM BODY WEIGHT)	ADRENALS (MG /100 GM BODY WEIGHT)	KIDNEY (MG /100 CM BODY SURFACE)
I, Control	21	+13	310 (270-370)	27 (23-30)	315 (286-343)
II, DOCA	20	+17	425 (380-480)	30 (21-37)	513 (426-573)
III, APP	32	+47	402 (250-540)	77 (40-99)	515 (328-613)
IV, DOCA + APP	17	-15	505 (352-608)	64 (38-83)	587 (318-683)

*Growth* As seen from Table I, all animals gained weight except those of Group IV which received combined treatment with DOCA and APP. In this group, weight loss was noticeable from the beginning of the experiment, the rats became shabby and asthenic, lost interest in food, and began to die around the twenty-first day, so that this experiment had to be terminated on the twentieth day. This effect is surprising because DOCA alone produced a slight inhibition and APP a stimulation of growth. Presumably as a result of anti-hormone formation, the growth hormone effect of APP was much more evident around the twenty-fifth day than on the sixty-first day.

*Diuresis* Each point on the curves of Fig. 1 represents an average of collections during a period of five days, so that individual variations are eliminated and the curves accurately portray the trend in each group. The increase in diuresis in Group II (DOCA) appears very early and reaches a maximum around the thirty-fifth day. There is no difference in diuresis between control and APP groups, urine formation in both increases slowly but regularly during thirty-five days and then levels off. It is interesting to note that maximum diuresis is reached at the same time as in the DOCA group. Quantitatively, the curve of Group II contrasts markedly with those of Groups I and III.

*Blood Pressure* Mean blood pressures with individual extremes for Groups I, II, and III are represented graphically in Fig. 2. Blood pressure in the controls was stable during the first six days, after which the trend was variable and slightly upward. The DOCA group showed a definite rise in pressure after the tenth day which reached a maximum at about the twenty-fifth day, at which level it persisted. It is to be noted that *all* the animals in this group became hypertensive. In the APP group, the shape of the curve was more irregular, because of the small proportion of hypertensive animals, it never reached values as high as in the preceding group. Only nine rats out of thirty-two in Group III had pressures higher than 150 mm Hg, and out of these nine, the two most hypertensive showed pressures of 190 and 195 mm Hg respectively. These last two rats died on the fortieth day, this accounts in part for the decrease in pressure mean after this time. In this group, pressures in some animals were always within the normal range. This contrasts with what occurred in the DOCA group.

We have not graphed here the blood pressures of Group IV (DOCA and APP) because this experiment terminated on the twentieth day. Also, the curve did not differ significantly from that of Group II. The mean blood

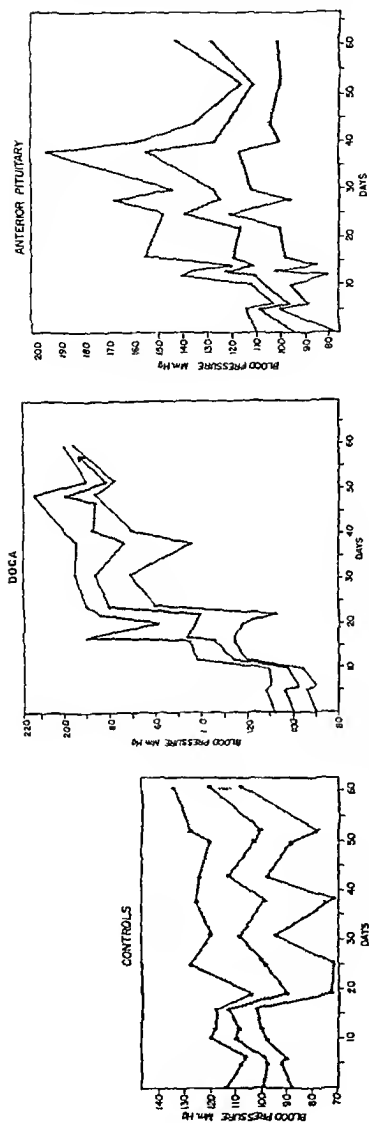


Fig. —Effect of desoxycorticosterone acetate and anterior pituitary preparations on blood pressure

pressures were day 0, 93 mm Hg, day 6, 115, day 12, 140, day 17, 147, and day 29, 161. Thus the combined treatment had no more effect on blood pressure than DOCA alone.

*Organ Weights* Heart weight was greater in all experimental groups than in the controls, but did not have any consistent relationship to blood pressure levels. Thus mean heart weights in Groups II and III were the same, although the terminal blood pressure averages were, respectively, 190 and 127 mm Hg. Similarly, kidneys were heavier in the experimental than in the control groups, although growth hormone was administered to Group III only. Adrenal weights were increased in Groups III and IV but unchanged in Group II. The absence of atrophy in Group II was probably due to the low dose of DOCA administered. The adrenal stimulation in Group III is consistent with a relationship between adrenocortical activity and high blood pressure; indeed, it was noticed that the animals presenting the most hypertension were those with the heaviest adrenals. However, closer examination of these glands frequently revealed hemorrhages and necrosis.

#### *Summary —*

From these experiments we see (1) with regard to blood pressure, that the effects of DOCA and APP differ significantly in that hypertension is more severe and much more consistent with DOCA than with APP, (2) concerning diuresis, the expected increase is observed with DOCA, administration of APP does not have this effect, (3) that combined treatment with APP and DOCA has no additive or synergistic effect and is in fact rapidly lethal. From this it seems that the mechanisms of the action of DOCA and APP are basically different. However, before concluding, the effects of APP must be examined in more detail.

#### II EFFECTS OF PARTIALLY PURIFIED ANTERIOR PITUITARY EXTRACT

With APP, the maximum rise in mean blood pressure was obtained around the twenty-fifth to the fortieth day and was followed by a decline. Also, a certain degree of resistance and adaptation occurred, evidenced by a decrease in body growth and ovarian weight, toward the end of the experiment. Therefore, it seemed possible that the effects of APP might have been altered by the action of antihormones. To evaluate this possibility, the following experiment was devised in which rats were given progressively increasing doses of pituitary hormones.

#### *Experimental —*

Female albino rats weighing between 106 and 138 grams (average, 124 gram) were fed a high protein, high sodium diet and were partially nephrectomized. Thirty-six animals were divided equally into three groups. Group I served as control, Group II received subcutaneously the suspension of anterior pituitary powder (APP), Group III received an alkaline anterior pituitary extract (APE). This last group was added in order to test the hypertensive properties of a partially purified anterior pituitary extract, since it has been claimed (Hay and Seguin, 1946) that such preparations cause less kidney damage than crude APP.

This extract was prepared by bringing a water suspension of lyophilized anterior pituitary powder to pH 9 with dilute NaOH. The mixture stood overnight at 10° C, it was

centrifuged the precipitate discarded and the pH of the supernatant adjusted to 7 with dilute acetic acid. The volume of this solution was adjusted so that 1 cc contained the equivalent of 50 mg of the dry powder.

Animals of Groups II and III received during the first thirteen days a daily dose of 90 mg of powder or its equivalent in extract in two injections; the dose was raised to 40 mg or equivalent until the twenty-third day and to 60 mg until the fiftieth day of the experiment at which time the animals were sacrificed and autopsied.

### Results—

Effects on blood pressure are summarized in Fig 3. In the control group again the blood pressure had a tendency to increase slightly during the thirteen days and then to remain relatively stationary. This confirms the observation of Grollman (1946) who noticed a rise in blood pressure following unilateral nephrectomy and administration of NaCl. The blood pressure curves of Groups II and III do not differ significantly as in the former series with APP the pressure rose rapidly during the first fifteen to twenty days then tended to level off. In contrast to our former series with APP, all of the animals showed some increase in pressure although many were no higher than in the control group. There were few animals with pressures higher than 150 mm Hg, of these there were two rats in Group II (154 and 176 mm Hg) and three rats in Group III (150, 185 and 186 mm Hg).

Organ weights are summarized in Table II. In accordance with the hypothesis that antihormone activity might be overcome by increasing doses of hormones, a sustained stimulation of body growth persisted to the end of the experiment with no difference between Groups II and III. Heart weights were not significantly different in the three groups. Kidneys were heaviest in Group II and heavier in Group III than in Group I. Stimulation of adrenal growth occurred only in Group II.

TABLE II COMPARATIVE EFFECTS OF CRUDE (APP) AND PARTIALLY PURIFIED (APE) ANTERIOR PITUITARY PREPARATION

GROUP	BODY WEIGHT		HEART (MG/100 GM BODY WEIGHT)	ADRENALS (MG/100 GM BODY WEIGHT)	KIDNEYS (MG/100 GM BODY SURFACE)
	INITIAL	FINAL			
I Control	127 (120-136)	183 (155-225)	320 (284-405)	23 (20-26)	290 (202-358)
II APP	123 (100-138)	237 (228-245)	330 (289-428)	33 (26-46)	456 (393-671)
III APE	124 (118-138)	239 (210-280)	346 (312-428)	25 (18-40)	384 (297-665)

The lack of adrenal stimulation in Group III (APE) can be explained partly by the removal of impurities from the original powder which would act as nonspecific damaging agents in Group II. The difference in the content in impurities between APP and APE was evident at autopsy: abscesses in subcutaneous tissues were present only in APP treated rats. Further, the relatively low content in adrenocorticotrophin of beef pituitary is well known. The comparative adrenal weights and blood pressures in Groups II and III show a clear dissociation of these functions. Furthermore increased adrenal weight does not imply increased function since the adrenals weighing more than 100 mg. were very often hemorrhagic or necrotic.

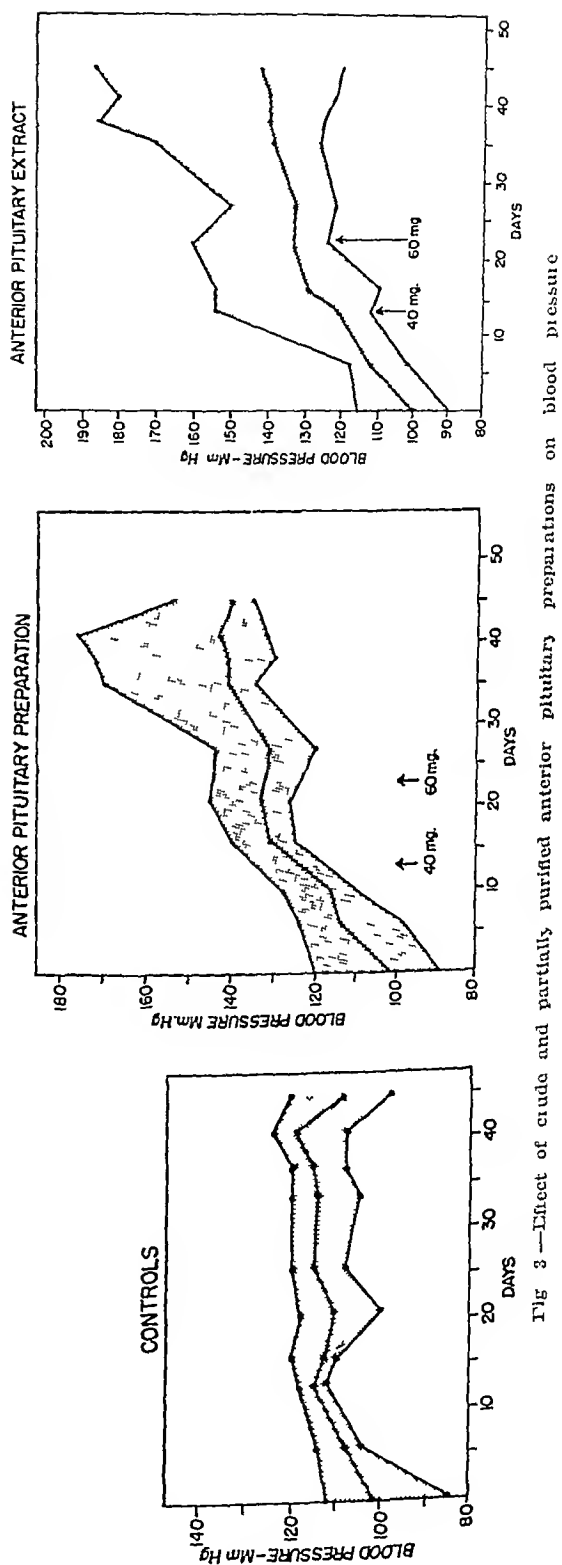


Fig. 3.—Effect of crude and partially purified anterior pituitary preparations on blood pressure



*Summary —*

These facts demonstrate a lack of relationship between adrenal growth and blood pressure level which contrasts with observations in animals given crude APP. It follows that the effects on blood pressure of APP may not be wholly the result of adrenal stimulation. Rather, with crude APP the changes in blood pressure and adrenals although concurrent may be unrelated.

## DISCUSSION

Hypertension following DOCA treatment has been observed in rats (Selye, Hall, and Rowley 1943; Green 1948; Friedman, Polley and Friedman, 1948). While Knowlton and co-workers (1946) have reported normal blood pressure in rats treated for ten weeks with 25 mg of DOCA daily and given 0.2 per cent saline solution as drinking water, this apparent inactivity can be attributed to an insufficient supply of sodium. In our experiments the same amount of DOCA plus 1 per cent saline solution uniformly produced severe hypertension within a period of three weeks. Actually, the amount of DOCA utilized was less than 25 mg. For at autopsy small deposits of crystalline material were still visible at the sites of injections. Hypertension can therefore be elicited by DOCA as long as the animals are given a high sodium diet.

Injections of anterior pituitary preparations stimulate physiologic body and organ growth when given in conjunction with a normal diet (Selye and associates 1945), however pathologic changes (hyalinization and enlargement of glomeruli, hyaline casts, arteriolosclerosis) occur if the animals are partially nephrectomized and maintained on a high protein and sodium diet (Selye, 1944). The latter changes are accompanied by a rise in blood pressure which in our experience seldom reaches the levels obtained following DOCA treatment, experimental perinephritis (Pige 1939), compression of the renal artery (Wilson and Byrom 1941) or partial renal infarction (Loomis 1946). Severe pathologic changes and hypertension are seen only in some of the experimental animals even though large doses are given to all. This is not in accord with the known dose responses of either steroid or protein hormones. The question arises: Are the pathologic effects of anterior pituitary hormones truly hormonal or do they represent nonspecific effects of foreign proteins or some combination of these actions?

Selye (1944) postulated from observations in rats that anterior pituitary preparations act through the adrenals by releasing DOCA-like compounds. However, a variety of observations made in rats tends to negate this view. (1) Recent studies indicate that it is the zona glomerulosa and not the cortex as a whole which supplies the body with DOCA-like compounds (Sarrison 1943; Greep and Denne 1947). (2) The zona glomerulosa does not atrophy following hypophysectomy (Smith 1930). (3) The activity of the glomerulosa depends in part at least on the Na/K ratio of the blood (Dane Shaw, and Greep 1948; Nichols 1948). (4) Anterior pituitary preparations and stress stimulate the inner zone of the adrenocortex which is considered

to be the site of production of glucocorticosteroids (Swann, 1940, Nichols, 1948) (5) There is no proof that glucocorticosteroids have blood pressure raising properties in normal animals (6) Adrenocorticotrophin and adrenal cortical extract tend to decrease blood pressure in hypertensive rats (Corcoran, 1948) Finally, (7) our present observations show a lack of similarity between DOCA- and APP-induced hypertension and a dissociation between adrenal weight and blood pressure level in rats given APE

Thus we recognize and confirm but cannot fully explain the hypertension caused by DOCA in rats That this may be a renal hypertension is suggested by its consistent association with renal lesions We also confirm the fact that hypertension can be produced in rats by injections of APP However, the view that this hypertension is caused by excessive secretion of DOCA like compounds from the adrenal is not supported by our data As to its mechanism, we again point to its association with renal lesions

The factors which produce these lesions are probably multiple In the rat, conditions such as high protein diets which greatly increase the renal functional load can result in morphologic lesions (Blatherwick and Medlar, 1937) The renal lesions caused by APP can thus be attributed to the combined effect of a diet rich in protein and sodium, which places a great functional demand on the kidney, and hormonal factors which stimulate renal growth (growth hormone, thyrotrophin, possibly a nephrotrophin\*) (Selye, Stone, Nielsen, and Leblond, 1945, White, Heinbecker, and Rolf, 1949) If it can be accepted that a cell does not function effectively during mitotic fission, it is clear that these superimposed factors will result in a relative renal insufficiency And, under other conditions of renal insufficiency, such as subtotal nephrectomy (Chanutin and Ludwig, 1939, Addis, Barrett, Lew, Poo, and Yuen, 1946) or serum nephritis (Smadel and Farr, 1939), high protein diets are known rapidly to precipitate morphologic regression Another mechanism of renal damage may be the foreign proteins contained in the pituitary preparations, since injections of proteins are known to cause degenerative renal lesions in rodents (Masugi and Sato, 1934, Oberling, 1943)

#### SUMMARY AND CONCLUSIONS

1 Desoxycorticosterone acetate (DOCA) and anterior pituitary preparation (APP) were tested in unilaterally nephrectomized female rats fed a high protein and a high sodium diet

2 DOCA (25 mg a day) elicited hypertension in all animals, APP had this effect in only a relatively small proportion of animals tested Combined treatment with DOCA and APP had no more effect on blood pressure than DOCA alone, rather, the animals rapidly sickened and died

3 DOCA caused severe diuresis, while control and APP treated groups showed only a slight increase in urine formation at the beginning of the experiment

\*We suggest this term as etymologically preferable to the term "renotrophin"

4 In rats treated with crude APP there is an association between hypertension and increased adrenal weights. But these large adrenals are often hemorrhagic or necrotic. The relationship of adrenal weight to blood pressure disappears when animals are treated with a partially purified APP extract.

5 The hypothesis that APP hypertension in rats depends on hypersecretion of DOCA like compounds thus appears unlikely.

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## REGRESSION OF ATHEROSCLEROTIC LESIONS ON CESSATION OF CHOLESTEROL FEEDING IN THE CHICK

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DESPITE the multitude of experiments in which cholesterol has been fed to many species in order to induce atherosclerosis little is actually known concerning the natural history of the lesions so produced. The observations recorded in the literature are few and fragmentary<sup>1,2</sup> based on small groups and in some instances on individual animals. In view of the large volume of work in the field of experimental atherosclerosis the need for a standard base line for comparison is imperative. With this end in view we have already reported on the relationship of atheromatosis to the amount of cholesterol added to the diet in the chicken.<sup>3</sup> We demonstrated a semiquantitative relationship between the per cent of cholesterol in the diet and the duration of feeding on the one hand and the severity of the resultant atheromatosis on the other. It was found that for concentrations of dietary cholesterol above 1/2 per cent there was no increase in the degree of atherosclerosis upon prolonging the feeding period from ten to fifteen weeks. We therefore decided to investigate the effects of cessation of cholesterol feeding on the hypercholesterolemia and atherosclerosis resulting from moderate periods of cholesterol feeding.

### METHODS

One hundred and fifty five 6 to 8 week old white leghorn cockerels were placed on a diet of chick starter mash enriched with 2 per cent cholesterol in 20 per cent cottonseed oil and fed ad libitum. In our experience when given for ten weeks this diet produces atherosclerosis in 100 per cent of chicks.<sup>3</sup>

After ten weeks of feeding the surviving cholesterol fed chicks were subdivided into three groups. Thirty one chicks continued to receive cholesterol for an additional fourteen weeks. Twenty five were placed on a diet of chick starter mash and the third group of twenty five received specially prepared mash from which the fat and cholesterol had been extracted by a commercial degreasing process. By this means the fat content of the mash was reduced from 4.5 per cent to 0.2 per cent and the cholesterol from approximately 100 mg per cent to 0. Vitamin supplements and sucrose were added to make this diet equivalent in caloric value to normal mash.<sup>4</sup> Twenty five control chicks were maintained on commercial chick starter mash throughout the entire experimental period.

Chicks were sacrificed at the conclusion of the ten week period of cholesterol feeding and at intervals of three to four weeks thereafter. Because of spontaneous deaths among the

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flock, it was not possible to adhere to a rigid schedule of sacrificing. However, attempts were made to obtain comparable samples from all the groups. All birds were autopsied, the organs inspected and the hearts and aortas dissected out en bloc, opened, and graded for extent and severity of atherosclerosis. A sketch of the lesions was made on a specially prepared form, and they were graded 0 to 4 on the basis of criteria in use in our laboratory.<sup>6</sup> The thoracic and abdominal portions of the aorta were graded separately but have been combined in our tables for simplicity in presentation. Tissues were preserved in formalin. Sections were taken from the most severely involved areas in the aorta of each bird, and wherever possible specimens were taken from both the thoracic and the abdominal aorta. Hematoxylin and eosin stained specimens and frozen sections stained with sudan IV were prepared and studied.

Blood was drawn from the alar vein for cholesterol determination by the method of Schoenheimer and Sperry.<sup>8</sup> Samples were taken at five and ten weeks after commencement of the experiment, and then at weekly and biweekly intervals until the conclusion of the experiment.

## RESULTS

*A Morphologic Observations in the Various Groups*—It seemed a worthwhile procedure to discuss the morphology in some detail because (a) this is the longest period of cholesterol feeding undertaken in the chick, along with an equally adequate group of controls for comparison, and (b) this is the first time, in a species other than the rabbit, that the opportunity has been afforded for observations over an extended period on the effects of cessation of cholesterol feeding on the arterial intima of the chick.

*Controls Receiving No Cholesterol Throughout Study* Of the four control chicks which showed gross lesions, one had a lesion of the thoracic aorta alone, two had lesions of the abdominal aorta, and one had lesions in both portions of the aorta. Of the gross lesions, those in the thoracic aorta were flat, nonraised, whitish or light yellow areas, varying in size from a few millimeters to 1 cm. in their greatest dimension. In the abdominal aorta the lesions appeared as a prominent ridge like area lying between the renal arteries on the posterior wall of the aorta. They are characterized by a light yellow staining of this area. Microscopic lesions were far more common. Nine out of twelve aortas from which frozen sections were made showed evidences of spontaneous atherosclerosis. The lesions have already been described fully by Dauber<sup>9</sup> and Charkoff and associates,<sup>10</sup> and we are in full accord with their findings.<sup>11</sup> Typical lipid deposits were found in the thoracic aorta in our cockerels. Microscopically the hematoxylin and eosin sections of thoracic aorta showed only scattered focal areas of loosely arranged ground substance which had almost the appearance of mucoid degeneration. These foci lay between the elastic and collagenous fibers of the intima and between the elastic laminae of the media. On frozen section, these areas were strongly sudanophile, and the stained material lay free in the ground substance (Fig 1, A). Charkoff and co-workers<sup>10</sup> have examined these deposits under polarized light and believe them to be free of cholesterol, containing only fat. Fatty material was most commonly found in the inner third of the media. The myocardium and coronary arteries were not involved in any way. The most striking changes were seen in the abdominal or muscular section of the aorta. As described by Dauber,<sup>9</sup> the primary lesions were to be found in the ridge like prominence in the interrenal region. The lesions are characteristically fibrotic, being made up of collections of collagenous bundles and fibroblasts, with small to moderate amounts of sudanophile material in the depths of the plaques, usually next to the intimal medial boundary. An excellent illustration of such a lesion is Fig 1, B.

*Birds Maintained on 2 Per Cent Cholesterol Diet Throughout* The earliest gross lesions were observed in the brachiocephalic vessels and the elastic aorta (referred to as the thoracic aorta). They appeared in the form of flat, nonraised, white or whitish yellow areas next to

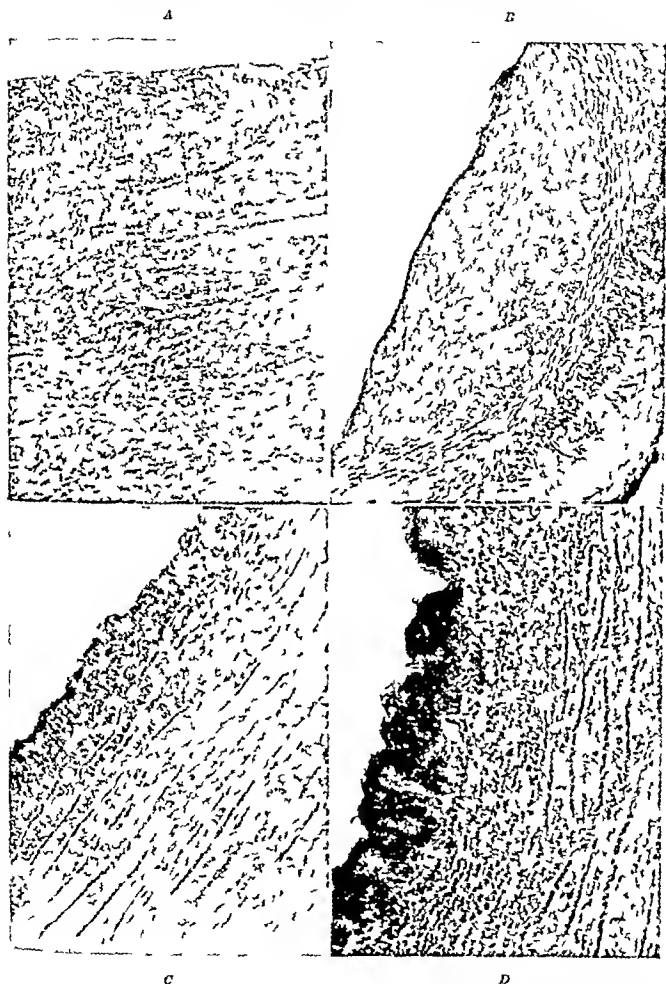


Fig 1—A Bird 2 control on normal mash diet twenty four weeks. Spontaneous lesion of the thoracic aorta. There is focal accumulation of sudanophilic material in the intima and media (note dark staining areas). (Frozen section  $\times 10$  sudan IV.) B Bird 37 control on normal mash diet fourteen weeks. Spontaneous lesion of the abdominal aorta. There is marked fibrous and fibroblastic thickening of the intima. Region near intimal medial boundary contains small amount of sudanophilic material. (Frozen section  $\times 10$  sudan IV.) C Bird 167 on cholesterol fourteen weeks. Early cholesterol induced lesion of the thoracic aorta. Note deposition of sudanophilic material in intima and inner portion of media. (Frozen section  $\times 10$  sudan IV.) D Bird 191 on cholesterol twenty four weeks. Very heavy deposit of sudanophilic material in the intima and inner one-third media. (Frozen section  $\times 10$  sudan IV.)

more than a few millimeters in diameter. This progressed to increasing thickening of the intima in these regions and the appearance of more extensive, more deeply pigmented patches, which were striated or nodular in gross appearance. Progression led to severe involvement of almost the entire elastic aorta by either a confluent plaque or separate and extensive nodular or granular plaques. There was a clear line of demarcation at the junction of the elastic and muscular aorta. The aortic valves were frequently involved with heavy plaques of yellow material in the sinuses of Valsalva. The mitral valves showed pin point lipid deposits in their substance. We again noted<sup>6</sup> that there is a definite time lag in the involvement of the abdominal portion of the aorta. The interrenal area is preferentially involved. Involvement ranged from slight yellow streaking to almost complete occlusion of the lumen of the aorta by massive deep yellow plugs. Isolated nodular involvement of the proximal portion of the iliac vessels was a common phenomenon.

Microscopically, the least severe lesions consisted merely of some increased deposition of gray blue ground substance between the collagenous and reticular fibers of the intima. On frozen section and sudan staining these areas contained moderate amounts of sudanophile material and an increased number of fibroblasts (Fig 1, C). Sudanophile material could also be seen scattered lightly through the inner half of the media. Most of the early lesions however consisted of foam cell plaques of varying degrees of severity—from a single layer of cells to veritable foam cell cushions, several layers deep. The fat filled fibroblastic cells were arrayed along vertically disposed reticular fibers and their nuclei also were perpendicular to the intimal coat. Usually foam cells and an increased amount of intercellular ground substance could be seen infiltrating the inner half to one third of the medial coat (Fig 1, D). The coronary arteries were relatively uninvolved in these early lesions. As the period of cholesterol feeding was prolonged, more severe changes were seen in the thoracic aorta. The progression is not clear in all instances, i.e., some lesions were seen at 140 days which, comparatively speaking, were no more severe than those seen at 70 days. However, a number of very severe lesions were seen late in the feeding period. Hyaline and cartilaginous metaplasia associated with breakdown of the foam cell plaques, appears to be the first step. With time there is a disappearance of the well formed foam cells, although ghost cells can be seen in the areas undergoing hyaline metaplasia. Typical cartilage does appear in the mid depth of the plaque, which eventually becomes heavily basophilic and in some instances undergoes extensive calcification. In some sections there is actual breakdown of the centers of the foam cell plaques with the formation of "abscesses" containing necrotic debris, fragments of nuclei, free fat, cholesterol crystal clefts, and heavy deposits of calcium in granules or platelets. In the heavier foam cell plaques which remain intact, numerous fine vacuoles may be seen communicating with the lumen, and endothelialized spaces were found resembling vasa which are filled with foam cells, loose and in clumps (Fig 2, A).

The media is involved in all instances, particularly in its inner half, by infiltration of foam cells and free fat. Where the intimal lesions are very severe, there may be severe attenuation of the underlying media. Involvement of the coronary arteries is common in association with severe aortic atheromatosis. Foam cell plaques may be seen partially or completely occluding the lumina of small arterioles. Some of the lesions also show a scattered calcium granule deposition. Fig 2, C shows a coronary arteriole severely narrowed by foam cells with a heavy plaque of calcium almost completely replacing the underlying media.

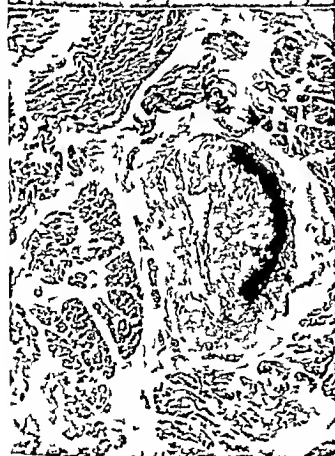
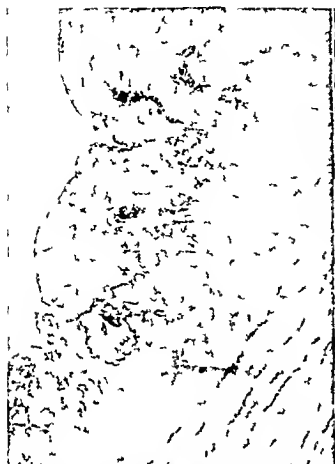
The thoracic aorta therefore shows a spectrum of changes extending from minimal deposits of fat and cholesterol in the ground substance to extensive foam cell plaques, atheromatous abscesses, hyalinization, cartilaginous metaplasia, and calcification.

There appeared to be little or no correlation between the severity of the lesions in the thoracic aorta and those in the abdominal aorta. Early lesions in the abdominal aorta were of two types. One group was indistinguishable from those of the "spontaneous" type which



A

B



C

D

FIG. 1. — A Bird 188 on cholesterol fifteen and one half weeks. Severe foam cell plaque of thoracic aorta. Note vascularization containing red blood cells and also the endothelialized spaces filled with foam cells discrete and in clumps. (X10 hematoxylin and eosin.) B Bird 169 on cholesterol thirteen and one half weeks. Advanced lesion of thoracic aorta. Note superficial layer of dense connective tissue beneath which is a wide zone of cartilaginous metaplasia with numerous foci of calcification. Involves inner one third of media as well as intima. (X10 hematoxylin and eosin.) C Bird 49 on cholesterol seventeen weeks. Severe narrowing of lumen of coronary artery by foam cells. There is a heavy plate of calcium almost completely replacing the underlying media. (X10 hematoxylin and eosin.) D Bird 191 on cholesterol twenty four weeks. Lesion of abdominal aorta shows superficial layer of dense fibrosis with much lipid and abscess formation in the depths of the plaque. (Frozen section X10 sudan IV.)

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Fig 3—A Bld 488 on cholesterol for fifteen weeks. Very severe lesion of abdominal aorta. Surface of lesion is coated by a thin layer of connective tissue beneath which lies a deep layer of ground substance intersected by fine collagenous strands and containing cholesterol crystals calcium granules and foam cell ghosts ( $\times 10$  hematoxylin and eosin.) B Bird 63 on chole terol for ten weeks and then on ordinary mash for fourteen weeks. There is very minimal thickening of the intima of the thoracic aorta which is fibrocellular in character ( $\times 10$  hematoxylin and eosin.) C Bld 97, on cholesterol for ten weeks, then on ordinary mash for fourteen weeks. Note globular sudanophilic masses immediately beneath endothelial lining of vessel. Also scattered sudanophilic granules in the remainder of the intima. (Frozen section  $\times 480$  sudan IV.) D, Bld 147 on chole terol for ten weeks then on special low fat diet for five weeks. Note atheromatous abscess cavity surrounded by fibrocellular connective tissue. (Frozen section  $\times 120$  sudan IV.)

and (2) more severe lesions showing heavy fibrosis but also severe residual deposits of sudanophile material and calcium. The latter probably represent the evolution of the second type of early abdominal lesion seen in the continuing cholesterol feeding. Fig 3, D is an illustration of one of these. It shows marked thickening of the intimal surface with fibrocellular connective tissue and partial obliteration by fibrous tissue of an atheromatous abscess. The cavities contain sudanophile material and heavy deposits of calcium. The underlying media shows some fibrosis.

In summary, cessation of cholesterol feeding and the institution of a low fat or normal mash diet were followed by fibrotic changes in lesions of both the thoracic and abdominal portions of the aorta, by the disappearance of foam cells, and by calcification of atheromatous abscesses. We also have noted the presence of scavenger-like cells in the intima.

*B Summary of Data on Degree of Atherosclerotic Lesions in Various Groups*—Table I summarizes the average gross gradings in the various groups for the entire duration of the experiment. The base line value of the severity of lesions at the end of ten weeks of feeding with cholesterol is based upon all chicks, both experimental and control, which either died or were sacrificed. Also included in this base line group are three birds from the low fat mash group and two from the plain mash group which died during the first week following division of the groups. The average gross grading for the eighteen chickens in this group was 2.5, which compares favorably with values reported by us in a previous experiment.<sup>6</sup>

TABLE I AVERAGE GROSS GRADING OF AORTIC ATHEROSCLEROSIS IN ALL GROUPS FOR ENTIRE DURATION OF EXPERIMENT

TIME FROM BEGINNING OF EXPERIMENT (WK)	ON 2% CHOLESTEROL DIET FOR 10 WEEKS AND THEN CONTINUED ON						CONTROLS ON PLAIN MASH	
	2% CHOLESTEROL		LOW FAT DIET		PLAIN MASH		NUMBER OF BIRDS	AVERAGE GRADE
	NUMBER OF BIRDS	AVERAGE GRADE	NUMBER OF BIRDS	AVERAGE GRADE	NUMBER OF BIRDS	AVERAGE GRADE		
9 to 11	18*	2.5					2	0
12 to 14	6	4.1	6	2.7	12	2.4	5	0.05
15 to 18	11	4.3	4	2.1	4	1.8	5	0.05
19 to 22	4	4.3	7	1.1	3	3.5	2	0
22 to 24	4	5.9	5	0.5	4	1.0	12	0.1
Total	43		22		23		26	

\*Includes seven chicks which died in the week prior to separation of the groups and three from the low fat and two from the plain mash groups which died during the first week after separation of the groups.

*Continued Cholesterol Feeding* This is best visualized in Fig 4. The average gross grade rose from 2.5 at the time of division (ten weeks) to 4.1 for the periods fifteen to twenty-two weeks, and then rose to 5.9 for the period twenty-three to twenty-four weeks. There was, therefore, an early rapid rise in severity of lesions, then a tendency to level off after fifteen weeks of cholesterol feeding, and finally a further rise after twenty-two weeks of cholesterol feeding.

*Normal Mash* Placing cholesterol-fed birds on a diet of normal mash resulted in a gradual decline in the extent and severity of the atherosclerosis.

This is best seen for the periods fifteen to eighteen weeks and twenty three to twenty four weeks where the average values obtained were 1.8 and 1 respectively. For the period eighteen to twenty two weeks a value of 3.5 was obtained. This average was constructed from data from three chicks one of which had very severe lesions. On the whole however the trend toward decreasing severity of the lesions was unmistakable.

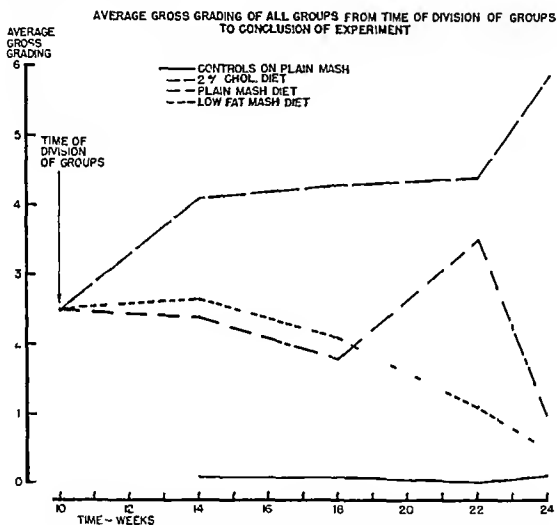


Fig. 4—Average gross grading of all groups from the time of division into groups to the conclusion of the experiment

*Low Fat* The group of cholesterol fed birds placed on a low fat cholesterol free diet gave values which closely resembled those obtained in the group of cholesterol fed birds placed on ordinary mash but the decline in the severity of the lesions was somewhat more marked and was consistent for all time periods. The decline first became evident at the fifteen to eighteen weeks period and was most noticeable during the eighteen to twenty two and twenty three to twenty four week periods where average gross values of 1.1 and 0.5 were recorded.

Combining the low fat and normal mash groups gave us a more adequate set of averages. Again a progressive decline in the severity of the lesions was apparent.

*Controls* The control group which received normal mash with no added cholesterol over the entire period was remarkable for the paucity of grossly

discernible atheromatous lesions of the so-called "spontaneous" variety. Only four of the twenty-six controls showed grossly visible atherosclerosis. Two were graded at  $\frac{1}{4}$ , one at  $\frac{1}{2}$ , and one at 1.

*C Blood Cholesterol Levels*—Fig 5 illustrates the blood cholesterol levels for the various groups throughout the course of the experiment. The first and second determinations were made at five and ten weeks after commencement of the experiment. Thereafter blood cholesterol determinations were

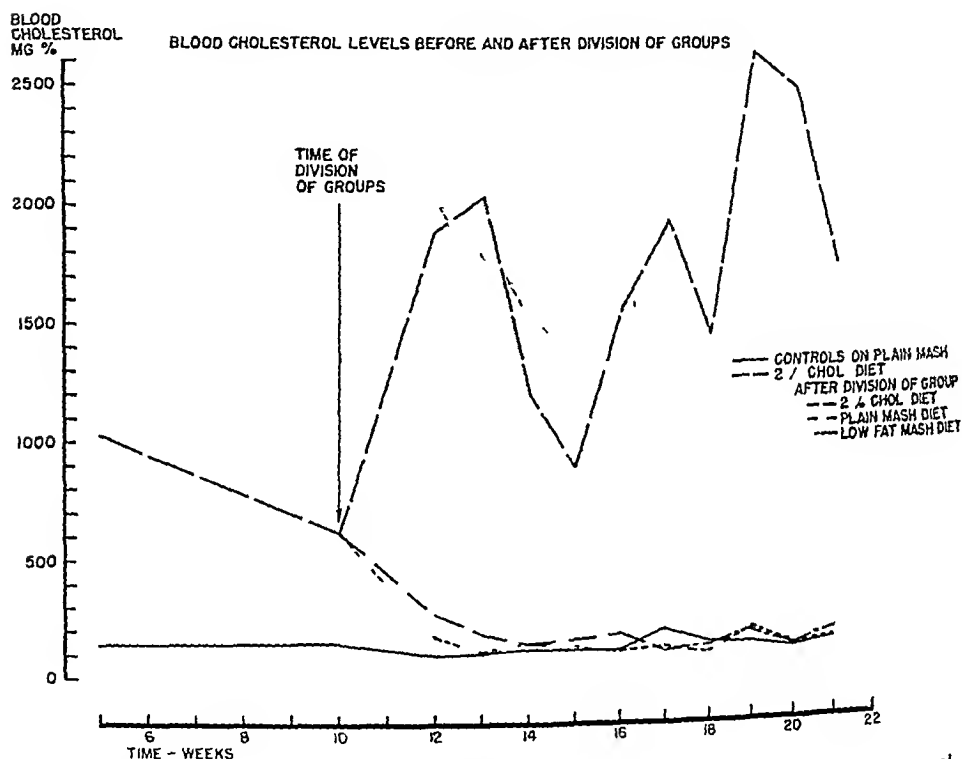


Fig 5—Average blood cholesterol levels before and after division of birds into the several groups

made at weekly or biweekly periods. The values for the 2 per cent cholesterol fed birds showed a good deal of variation from week to week, but the trend on the whole was toward increasing levels. The last three determinations were 2,629, 2,479, and 1,839 mg per cent respectively. During the same period the blood cholesterol level fell in the groups taken off the cholesterol diet. Within two weeks of cessation of cholesterol feeding, the blood cholesterol levels of the plain mash and low fat groups fell to 268 and 175 mg per cent respectively (control, 100 mg per cent). Within one more week the values for both groups were back in the normal range. The blood cholesterol levels of the controls showed no significant variation during this period.

In summary, continued cholesterol feeding after ten weeks results in the maintenance and further elevation of the blood cholesterol levels, while cessa-

tion of cholesterol feeding leads to a very rapid fall of the blood cholesterol back to normal levels

#### DISCUSSION

The results obtained in the present study complement and extend our previous studies on cholesterol induced atherosclerosis in the chicken. It is now clear that continuing cholesterol feeding in the chick for periods up to six months results in progressive aggravation of the atherosclerosis. This is not in accord with previous data reported by us for a much shorter experimental period.<sup>8</sup> The progression is associated with a continued rise in the blood cholesterol levels. The spectrum of microscopic findings resulting from continued cholesterol feeding is wide indeed ranging from mere increases in amorphous ground substance to massive foam cell plaques, hyaline and cartilaginous metaplasia and heavy calcium deposits. We noted also the tendency for cholesterol induced lesions of the abdominal aorta to appear in an area usually involved in the 'spontaneous' variety of the disease and apparently to accelerate and/or aggravate the normally occurring lesion.

Cessation of cholesterol feeding after ten weeks resulted in a gradual decline in the severity of the lesions as judged by gross grading in both the plain mash and low fat groups. There was no essential difference between the two groups either from a gross or microscopic standpoint, and they have been combined for purposes of discussion (and in Table I). Blood cholesterol levels fell to normal within three weeks of cessation of cholesterol feeding. Microscopically the lesions were characterized by diminution and disappearance of foam cell deposits, increasing fibrotic changes in both the abdominal and thoracic portions of the aorta, diminution of grossly stainable lipid, and heavier calcification. Some of the chickens sacrificed at the very end of the experiment showed so little gross or microscopic evidence of atherosclerosis that it may be assumed that the lesions in these birds had undergone complete remission. In other birds severe fibrosis, remains of atheromatous abscesses, and heavy calcification tended to suggest that there is a limit to the degree of atherosclerotic damage which can be repaired.

Controls kept on ordinary mash showed only few gross lesions, but the incidence of microscopic lesions was much higher. This is in accord with previous experience in this laboratory.<sup>9</sup> In corroboration of Chaikoff and co workers,<sup>10</sup> we found lipid deposits in the thoracic aortas of cockerels, a phenomenon reported by Dauber<sup>9</sup> only in hens. These lesions of the thoracic aorta are never characterized by thickening of the intima or by foam cell deposits but consist of small focal deposits of lipid material in the ground substance of the intima and inner portion of the media. In the abdominal aorta, the spontaneous lesions are characteristically fibrotic with far less stainable lipid than in the cholesterol induced lesions. They are, however, very similar to some of the less severe lesions induced by cholesterol feeding particularly when the latter has been suspended for some time and the chicks have been returned to a normal diet.

Other investigators have made observations on the effect of suspending cholesterol feeding on the arterial lesions of the rabbit. Stuckey,<sup>1</sup> Kiylov, Wada,<sup>2</sup> and Scarff<sup>3</sup> were the first to report such observations, but their data is not very extensive. Amitschkow<sup>4</sup> was the first to study the problem extensively in the rabbit. He found that following three to four months of cholesterol feeding, and the resumption of a normal diet, there resulted a gradual loss of lipids from the plaques. The latter lost their yellowish color and no longer stained with sudan. They presented a whitish and fibrous appearance. He stressed, however, that this process was a slow one. The transformation of a large plaque rich in lipids into a plaque composed of fibrous tissue took two to three years, and even then small quantities of lipid could be discovered microscopically after sudan staining. Microscopically, Amitschkow noted the gradual disintegration and disappearance of the foam cell masses and their replacement by newly developing collagenous and elastic fibers and fibroblasts. He noted also the tendency for cholesterol crystals to persist long after the other lipids have been removed, and also the large quantities of calcium which were laid down in areas formerly occupied by atheromatous abscesses. He stated that if the development of the lipid deposits and the thickening of the intima had not reached a very considerable degree during the progressive period, all that was to be seen after resorption of the lipids had taken place was a slight fibrous thickening of the intima, sometimes containing a few cholesterol crystals, together with scattered lipid cells and globules of neutral fat. Amitschkow gave no data on the effect of cessation of cholesterol feeding on the blood cholesterol levels in the rabbit. Stenel,<sup>5</sup> among others, has observed that following the cessation of cholesterol feeding in rabbits the blood cholesterol levels fall very slowly over a period of months. It is of interest that the resorptive changes seen in the rabbit, and described by Amitschkow and others, so closely resemble the changes seen by us in the chicken. It is also of interest that the rate of regression of lesions in the chicken is much greater than in the rabbit. These two features may well be related in an etiologic and pathogenetic sense, as will be discussed.

The ability of the physiologic mechanisms to remove lipids from the arterial wall is not a feature common to the experimental animal alone. In man, Leary's<sup>12</sup> excellent studies have thrown considerable light on the problem of the bodily defenses against the development of atheromatous lesions. Leary believes that a defense mechanism exists which removes excess fat and cholesterol from the arteries of youth and from the ascending aorta even in old age. The cholesterol is transferred from wandering lipid cells (foam cells) to fixed fibroblasts in which the cholesterol esters are split, anisotropism is lost, and the cholesterol is brought into solution in an excess of fatty acids. Solution is followed by its disappearance from the lesions. With the removal of the excess cholesterol, the lesions stop progressing toward atherosclerosis and subside. In youth this mechanism is adequate to cope with lipid deposits in the intima. As man ages his ability to remove cholesterol appears to diminish, and atherosclerosis becomes severe. Increased deposition of collagen



and dense scar tissue with subjacent collection of fat is more common. In old age, cholesterol removal mechanisms appear to cease and foam cells accumulate in masses with inadequate nutrition and support and primary atheromatous abscess is the typical lesion. Further corroborative evidence on the resorbability of human lesions comes from two sources. Aschoff<sup>14</sup> observed that atherosclerotic lesions were rare in autopsy material during and after World War I in Germany—a period of severe famine and malnutrition, especially with respect to fats, cholesterol and proteins. More recently Wilens<sup>15</sup> has noted that at necropsy severe atherosclerosis is at least twice as common in obese as in undernourished persons 35 years of age or over. He found this relationship to be independent of sex or the presence or absence of diabetes or hypertension. Severe or even moderate atherosclerosis was seldom found in those with protracted undernutrition. Less severe atherosclerosis was observed in those with wasting disease than in those with terminal weight loss. He inferred from this data that resolution of atheromatous lesions may occur during periods of marked weight loss. Histologically the most conspicuous difference between the two groups lay in the amount of lipid in the intimal lesions—there were less lipids and fewer phagocytes in the intimal lesions of those with weight loss.

Thus far we have discussed only the spontaneous regression of lesions which takes place in the experimental animal on the cessation of cholesterol feeding, and in man following prolonged undernutrition. We have already noted that the provision of a low fat cholesterol free diet did not appreciably affect the rate of regression of lesions in the chick. Attempts have been made to influence the rate of regression of lesions in the rabbit through the use of various substances known to influence fat metabolism. Steiner<sup>16</sup> noted that the administration of choline to rabbits which had received cholesterol for 110 days resulted in a marked regression of the lesions. Examination of his data reveals that in the six rabbits in which choline appeared to have a positive effect, the blood cholesterol levels were never very high and rapidly fell to the normal range e.g.

DAYS	0	20	60
	476 mg % 512	149 mg % 357	96 mg % 125

Where choline failed to influence the lesions the blood cholesterol was high at the start and tended to stay high e.g.

DAYS	0	60
	1265 mg % 000	1250 mg % 544

In the group taken off cholesterol and maintained on a regular diet without choline the cholesterol values with one exception were over 300 mg per cent at the end of sixty days and the rate of fall was very slow. It would seem therefore that there is a strong correlation between the rate of fall of

the blood cholesterol levels and the efficacy of choline in affecting the regression of lesions. The failure of choline to act in four out of ten rabbits might well be accounted for on the basis of individual differences among rabbits, which are known to respond in varying fashion to cholesterol feeding. Steiner's results have been amply confirmed by Morrison and Rossi.<sup>16</sup>

By means of cholesterol tolerance tests<sup>17, 18</sup> we have been able to show that there is a marked difference in the response of different species to a standard load of their homologous cholesterol administered intravenously. We have found that in comparison with the chicken and the rat, the rabbit is much less capable of dealing with intravenously injected cholesterol and requires three to four times longer to dispose of an equivalent load of cholesterol. Kendall<sup>19</sup> and associates have published similar data using an artificially prepared emulsion of cholesterol. It appears therefore that the ability of any species to remove cholesterol from the arterial intima is related to its ability to metabolize cholesterol. Thus the chicken, which removes injected cholesterol from its blood stream about three times as fast as the rabbit, also shows a very rapid fall of blood cholesterol to normal after cessation of cholesterol feeding and a more rapid regression of lesions than does the rabbit. That this process may be accelerated by exogenous factors is well demonstrated by the effect of choline in the rabbit, and by undernutrition in man. The existence of a physiologic mechanism for the removal of fat and cholesterol from the vascular wall is well supported by the evidence cited and is clearly suggested by the results of the present investigation. We are therefore compelled to abandon our "static" concepts concerning the nature of atherosclerosis and to apply instead the dynamic principles of modern metabolic theory concerning the behavior of the lipids in other parts of the body. Body fat is now known to be in a constant state of flux, it is laid down in depots and is constantly moved and replaced, converted or destroyed as required.<sup>20</sup> It would appear from the present study that under certain circumstances the lipid content of atheromatous plaques is subject to similar metabolic forces. Further studies on this aspect of the problem with more refined metabolic tools are the *sine qua non* of therapeutic researches in this field.

#### SUMMARY

1 Prolonged feeding of a diet containing 2 per cent cholesterol in cotton seed oil for a period of twenty-four weeks resulted in progressive elevation of the blood cholesterol levels. There was increasing severity of the atherosclerosis for a period of fifteen weeks and then a levelling off, with a further increase in severity during the last two weeks of the experiment.

2 Cessation of cholesterol feeding after ten weeks is followed by a very rapid decline in the blood cholesterol levels to normal within three weeks. There was also a gradual regression in the severity of the lesions over a fourteen-week period. It appears that early lesions may be completely resorbed upon cessation of cholesterol feeding, while more severe lesions undergo regressive and reparative changes.

3 There appeared to be little difference in the rate of regression or disappearance of the aortic lesions in birds placed on a normal mash diet and those placed on a low fat, cholesterol free diet following cessation of cholesterol feeding

4 There is a wide spectrum of histologic changes resulting from prolonged cholesterol feeding, ranging from increase in the ground substance of the intima, with infiltration of sudanophile material, to very extensive foam cell plaques hyaline and cartilaginous metaplasia and heavy deposits of calcium in granules and plates

5 Cessation of cholesterol feeding is followed by fibrotic changes in lesions of both the thoracic and abdominal portions of the aorta by the disappearance and diminution of foam cells and fat, and by the calcification of atheromatous abscesses. We also noted the presence of scavenger like, fat filled cells in the intima

6 The controls showed few gross lesions but numerous microscopic ones. Scattered focal deposits of sudanophile material were seen in the intima and inner portions of the media of the thoracic aorta. In the abdominal aorta the spontaneous lesions were characterized by fibrosis of the intima, with sudanophile deposits and calcium granules at the intimal medial junction

7 The presence of a physiologic mechanism concerned with the regression of atheroma in animals and man is discussed and the application of modern dynamic principles of lipid metabolism to this mechanism is considered

We are indebted to Miss C Bolene (D V Dauber Memorial Research Assistant), Miss Lorraine Adams, Miss Eileen Arnold, Miss Marilyn Dudley and Mr William Foote for technical assistance in this study

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## THE LACK OF EFFECT OF TWEEN 80 ON THE ABSORPTION OF ALUMINUM AND SODIUM PENICILLINS

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THE widespread use of oral penicillin despite the fact that only a fraction of an administered dose is absorbed from the gastrointestinal tract makes desirable the investigation of factors that may favor the absorption of penicillin from the gut. The present study reports the comparative plasma concentrations of penicillin that resulted from the oral administration of aluminum\* and sodium penicillins with and without orally administered Tween 80 (polyoxyethylene sorbitan monooleate).

The Tweens are a series of polyoxyalkylene derivatives of the hexitols mannitol and sorbitol, and their anhydrides partially esterified that are classified as dispersing agents.<sup>1</sup> Such compounds might theoretically increase the absorption of substances from the gastrointestinal tract by lowering surface tension and increasing dispersion. It has been reported that one of these compounds, Tween 20, given by mouth and parenterally prolonged the penicillin plasma concentrations following oral and parenterally administered penicillin.<sup>2</sup> Tween 80 differing only in the fact that oleic acid is esterified with the hexitan in place of lauric acid, has been fed to human patients in amounts as large as 15 Gm per day without evidence of toxicity and furthermore has proved to be effective in increasing the absorption of fats and fat soluble substances in certain pathologic conditions marked by impaired gastrointestinal absorption.<sup>3</sup> Because of its nontoxicity and its proved efficacy in human beings Tween 80 was chosen for this study to determine whether it had any influence on the absorption of orally administered penicillin.

### MATERIALS AND METHODS

Twelve male subjects varying in age from 33 to 48 years afebrile and free of obvious renal, hepatic or gastrointestinal dysfunction were elected from the medical wards and divided into two groups of six each. One group was given aluminum penicillin with and without Tween 80 and the other was given sodium penicillin with and without Tween 80 each patient serving as his own control in determining the effect of the dispersing agent on penicillin plasma concentrations.

Penicillin was administered orally in a dose of 200,000 units every three hours for nine doses and blood samples were drawn into heparin wetted syringes at one-half, one, two, and three hours after the ninth dose. No medication was given for twenty-four hours and then 100,000 units of penicillin and 2 Gm of Tween 80 were given together every three hours for nine doses. After the ninth dose of the combination of drugs, blood samples again were

From the Philadelphia General Hospital.

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Tween 80 was supplied through the courtesy of Atlas Powder Co., Wilmington, Del.; aluminum penicillin through the courtesy of Hynson, Westcott & Dunning, Inc., Baltimore, Md.; and sodium penicillin through the courtesy of Sharp & Dohme, Inc., Kenilworth, Pa.

drawn at one half, one, two, and three hours. The ninth dose of medication in each phase of the study was given at the same time of day and in the same relationship to the ingestion of food, so that the penicillin time dose response curves in the two phases of the study can be compared to determine the effect of Tween 80. The same lots of aluminum and sodium penicillins were employed throughout the study. Penicillin assays were done by the Kirby Rantz modification of the Rammelkamp serial dilution method,<sup>4</sup> employing as the test organism *Streptococcus* 98.

### RESULTS

In Table I are presented the data obtained following the use of sodium penicillin. It is apparent that in three patients (E U, B L, and P L) the penicillin plasma concentrations were actually lower when Tween 80 was administered, in two patients (G J and J M) the concentrations were higher, and in one patient (J M) the concentrations were equal to those obtained when penicillin was given alone. The average values from the six patients show the concentrations after penicillin alone to be slightly higher than those after penicillin and Tween 80.

TABLE I SODIUM PENICILLIN 200,000 UNITS\* ORALLY, COMPARISON OF PENICILLIN PLASMA CONCENTRATIONS WITH AND WITHOUT TWEEN 80

PATIENT	AGE	TWEEN 80†	PENICILLIN PLASMA CONCENTRATIONS (HOURS AFTER ADMINISTRATION)			
			½	1	2	3
G J	67	Without	0.19	0.19	0.38	0.25
		With	0.76	0.76	0.76	0.76
J M	78	Without	1.00	1.50	1.50	1.50
		With	2.00	1.00	1.50	1.00
J M	33	Without	0.25	0.19	0.25	0.13
		With	0.76	0.50	0.19	0.13
E U	72	Without	1.50	1.50	1.00	1.00
		With	---	0.38	0.38	0.25
B L	36	Without	0.97	1.65	1.25	0.95
		With	0.47	0.80	0.37	0.30
P L	45	Without	0.55	1.20	0.32	0.19
		With	0.30	0.55	0.25	0.17
Average		Without	0.74	1.03	0.78	0.67
		With	0.85	0.66	0.57	0.43

\*Two 100,000 unit tablets each buffered with 0.35 Gm. calcium carbonate.

†10 Gm. per day administered as four 0.5 Gm. gelatin capsules every three hours.

Table II shows similar data after the use of aluminum penicillin. In two patients (H M and C B) the plasma concentrations of penicillin are lower after the use of Tween 80, in two patients (J W and R M), slightly higher, and in two patients (S B and E H), approximately equal to those observed after the use of aluminum penicillin alone. The average values from the six patients are practically identical.

### DISCUSSION

The Tweens are painful and irritating when injected intramuscularly, so even if effective in elevating penicillin plasma concentrations as claimed for Tween 20,<sup>2</sup> this route of administration would be impractical. Furthermore the amount of Tween 80 absorbed from the gut and excreted by the kidneys is

TABLE II ALUMINUM PENICILLIN 200 000 UNITS ORALLY, COMPARISON OF PENICILLIN PLASMA CONCENTRATIONS WITH AND WITHOUT TWEEN 80

PATIENT	AGE	TWEEN 80†	PENICILLIN PLASMA CONCENTRATIONS (HOURS AFTER ADMINISTRATION)			
			½	1	2	3
J W	68	Without	1.60	1.60	1.60	1.60
		With	1.60	3.02	2.50	2.50
R M	67	Without	0.25	0.50	0.25	0.25
		With	0.51	0.76	0.76	0.25
S B	50	Without	0.19	0.19	0.25	0.25
		With	0.25	0.38	0.19	0.15
H M	59	Without	1.00	2.00	0.76	0.38
		With	0.76	0.76	0.50	0.76
C B	78	Without	3.00	3.00	3.00	1.50
		With	1.50	0.76	2.00	1.50
E H	71	Without	0.15	0.78	0.70	0.80
		With	0.41	0.98	0.73	1.20
Average		Without	1.05	1.34	1.05	0.79
		With	0.81	1.11	1.11	1.05

Four 50 000 unit tablets each buffered with 0.3 Gm sodium benzoate  
 †10 Gm per day administered as four 0.5 Gm gelatin capsules every three hours

so small<sup>5</sup> that a caramide like effect ascribed to Tween 20<sup>2</sup> seems unlikely. The sole anticipation of an effect of Tween 80 on the absorption of penicillin was predicated on its activity as a dispersing agent within the gut. The observations made in twelve patients under the conditions outlined indicate that Tween 80 exerted no effect on the absorption of penicillin from the gastrointestinal tract.

Aluminum and sodium penicillin were not compared in the same patients, but it is apparent that the penicillin plasma concentrations observed in similar groups of patients were approximately equal during the three hour period studied. A subsequent investigation will be directed toward comparing the maintenance of penicillin plasma concentrations for periods longer than three hours. It should be noted that the penicillin plasma concentrations were unexpectedly high, except initially, they exceeded those resulting from the intramuscular injection of 50,000 units of penicillin (crystalline penicillin in aqueous solution)<sup>6</sup>. The concentrations observed may be explained in part by the ages of the patients studied or by the possibility of penicillin accumulation in the circulation during the twenty four hours of medication prior to the time dose response values here recorded. Unfortunately there is no body of data in the literature with which to compare the results of this study but it would be unwarranted to assume that penicillin plasma concentrations of the magnitude herein reported obtain in all patients to whom 200,000 units of penicillin are orally administered every three hours.

#### CONCLUSIONS

Tween 80 (polyoxyethylene sorbitan monolaurate) in oral doses of 16 Gm per day did not enhance the gastrointestinal absorption of penicillin as determined by the penicillin plasma concentrations resulting from the oral administration of the antibiotic with and without Tween 80.

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## LABORATORY METHODS

### EVALUATION OF A MODIFIED SUMNER'S METHOD (DINITROSALICYLIC ACID) FOR DETERMINATION OF GLUCOSE IN URINE

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SUMNER<sup>1, 2, 3</sup> has described methods for the determination of glucose in urine and blood, using dinitrosalicylic acid as an oxidizing agent. The reduced dinitrosalicylic acid is determined colorimetrically. This method has been employed extensively by Exton<sup>4</sup> for the determination of both urinary glucose and by modification, other sugars found in urine. Recently Leech and Woodford<sup>5</sup> have utilized this principle in a simple and rapid method for the approximate estimation of blood glucose.

The present paper deals with the employment and detailed evaluation of this method slightly modified for the determination of glucose in urines that do not contain other reducing sugars.

#### PROCEDURE

The following procedure was found to be satisfactory:

*Preparation of the Reagent*—Solution 1. 120 Gm. of sodium potassium tartrate and 6 Gm. of phenol (crystalline) are dissolved in 300 cc. of water. A solution of 6 Gm. of sodium bisulfite in 60 cc. of water is added.

Suspension 2. 20 Gm. of 3.5 dinitrosalicylic acid monosodium salt are suspended in 800 cc. of water care being taken to avoid large lumps.

Solution 3. 40 Gm. of sodium hydroxide are dissolved in 400 cc. of water and cooled.

Forty cubic centimeters of Solution 3 are added to Suspension 2 and the mixture is shaken until the salt has dissolved. This is completed in a very short time if there are no large lumps in the suspension. Now Solution 1 is added and mixed well with the solution of dinitrosalicylate. Finally 320 cc. of Solution 3 are added in three or four portions. The mixture is shaken after the addition of each of these portions. If all of the sodium hydroxide is added at once, a precipitate may be formed which is very difficult to redissolve. The volume is made up to 2,000 cc. with water.

This reagent is kept for two weeks at 25 to 30° C. before use.

*The Reduction and the Colorimetric Determination*—Ten cubic centimeters of reagent are pipetted into a 19 mm. test tube and 0.1 cc. of urine is added from a blood capillary pipette. The pipette is rinsed in the reagent and the mixture is stirred with the pipette on a glass rod. To another 10 cc. of reagent in a similar test tube is added 0.1 cc. of water. The tubes are heated in boiling water for three minutes and cooled to room temperature.

The optical density is determined by spectrophotometry\* in a 19 mm. tube using the reagent blank as a standard. For glucose concentrations below 0.6

\*From the Department of Medicine, University of Chicago.

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Different type of colorimeter may be used. The described procedure is based upon the employment of a Coleman Junior Spectrophotometer which gives a very satisfactory result for this purpose. It may be operated rapidly and gives a reasonable degree of accuracy.

per cent the wave length  $540\text{ m}\mu$  is used, for concentrations above this value,  $700\text{ m}\mu$  is used. The glucose concentration is found from a standard curve or from Table III. Finally, the temperature of the solutions is measured and, if necessary, a correction made by means of Table IV.

#### RESULTS AND COMMENT

It was found that the results vary, depending on the method of preparing the reagent. The amount of color developed is further dependent upon the age of the batch of reagent used. From Fig. 1 it will be seen that the intensity of

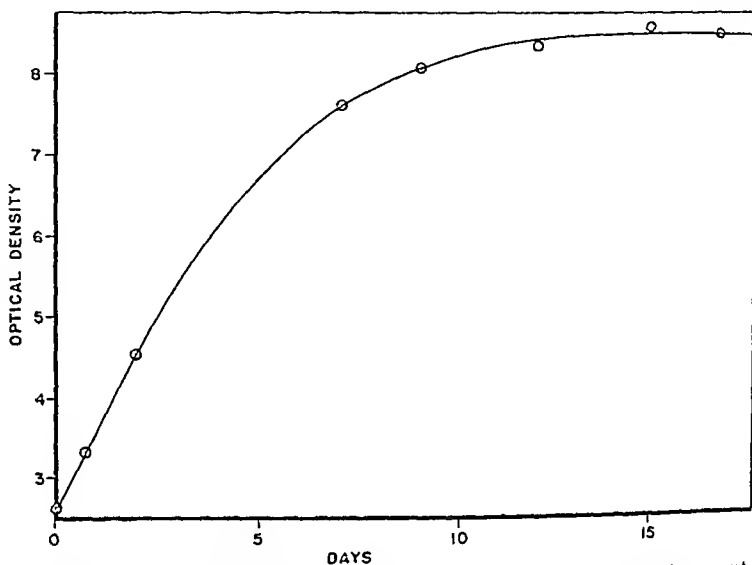


Fig. 1—The density of color obtained after boiling using the same batch of reagent at different periods of time after preparation. Constant glucose concentration.

the color, when the reagent is kept at  $27^{\circ}\text{C}$ , increases for the first twelve days or so and thereafter remains constant. It is thus necessary to leave the freshly prepared reagent for about two weeks at this temperature before use. At lower temperatures a longer time is required, while at  $37^{\circ}\text{C}$  the aging probably will be complete in one week. We have tried to age the reagent in five hours in boiling water. This procedure, although suitable for emergency use, is not recommended, since it results in a rather dark colored reagent which is unsuitable for exact work. As shown in Table I, the reagent, once it has been aged, is stable for at least three months at room temperature when kept in a brown bottle in the dark.

TABLE I DENSITIES FOUND, USING DIFFERENT BATCHES OF REAGENT  
All Glucose Concentrations Are 0.5% Each Value Is the Average of 5 Determinations

DATE OF PREPARATION OF REAGENT	AGE OF REAGENT (DAYS)	DENSITY AT $22^{\circ}\text{C}$
3/6	34	0.800
3/6	77	0.826
3/6	99	0.794
4/16	15	0.840
4/16	37	0.820
4/16	58	0.798
5/24	13	0.817
5/24	20	0.804

For pipetting the reagent an automatic 10 c.c. pipette\* is convenient

In these experiments 0.1 c.c. of urine† was used. The pipetting of this volume with a 0.1 c.c. capillary blood pipette is the largest single source of error in the method. The pipettes used in this work (Sargent Chicago), as received from the manufacturer, are said to measure 0.1 c.c.  $\pm$  0.005 cubic centimeter. All of thirty pipettes tried were found to be within these limits.

When working with pure glucose solutions or with urines containing much glucose and only small amounts of other reducing substances the mixture of urine and reagent may, if desired, be left at room temperature for an hour before boiling. Table II shows the values for optical density where different periods of time before boiling were employed‡. In the case of very concentrated solutions (10 per cent glucose) a certain amount of the brown color develops during the first hour at room temperature. This does not affect the final result.

TABLE II. VALUES FOR OPTICAL DENSITY FOUND WHEN A MIXTURE OF A PURE GLUCOSE SOLUTION AND THE REAGENT REMAINED AT ROOM TEMPERATURE FOR DIFFERENT PERIODS OF TIME BEFORE BOILING

TIME (MIN.)	DENSITY
0	0.630
5	0.64
10	0.652
30	0.653
60	0.642

If large amounts of creatinine are present reduction takes place at room temperature within a short time as indicated in Fig. 2. The amount of color developed by physiologic concentrations of creatinine is rather small and usually can be neglected. If one, however, is interested in determining concentrations of glucose in the range of 0.1 to 0.4 per cent the reduction by creatinine must be taken into consideration and the tubes should be put into the boiling water as soon as possible after mixing urine and reagent.

A boiling time of three minutes was found satisfactory. The variation of the color obtained by varying the boiling time from 2.5 to 4 minutes is seen in Fig. 3. One should make sure that there is proper contact between test tubes and boiling water. If many tubes are placed in the water bath without sufficient space between them, the tubes in the middle will remain at a lower temperature than the others and erroneous results will follow.

After boiling, the tubes are placed in cold water. It is convenient to have two vessels for this purpose. The tubes are first placed for a few minutes in one with running tap water and thereafter in the other containing water at room temperature. In this way rapid cooling is obtained and all the tubes will be at room temperature for the colorimetric determination. As will be shown later, this is essential since the intensity of the color depends upon temperature.

If mouth pipettes are used it must be remembered that the reagent is caustic and poisonous.

†It is usually unnecessary to filter the urine. Even urines containing large amounts of precipitates will usually give clear solutions on boiling in the strongly alkaline reagent.

‡The use of 19 mm test tubes for heating the mixture of reagent and urine in boiling water obviates any appreciable loss by evaporation.

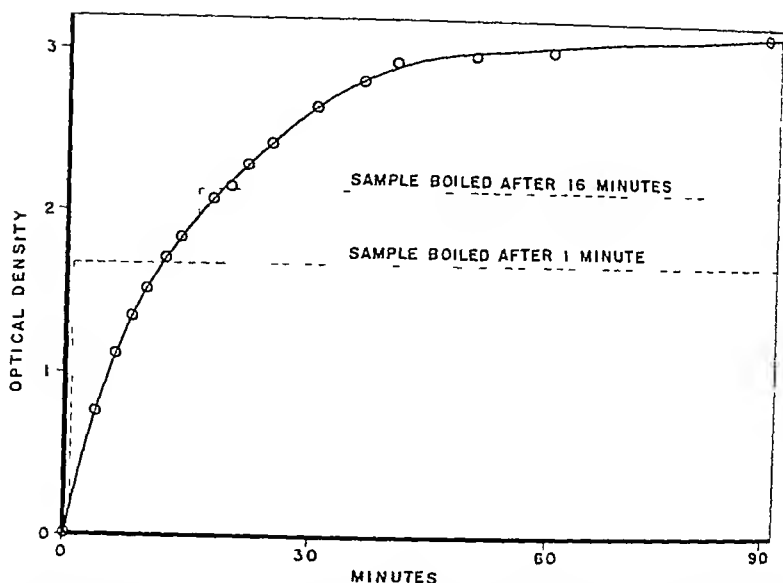


Fig 2—Development of color expressed as density at 540  $m\mu$  by a solution of creatinine corresponding to 3.8 per cent in 0.1 cc of urine added to the reagent and standing at 25° C. Two samples were taken out and boiled and thereby the development of color was stopped at a lower density.

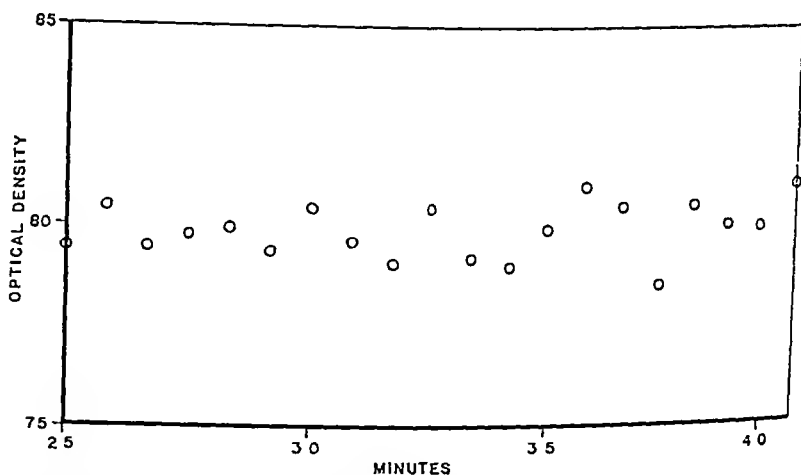


Fig 3—Twenty test tubes containing samples of the same mixture of glucose and reagent were boiled for different periods of time. The variation in density determined at 540  $m\mu$  after cooling reflects the statistical uncertainty of the determination. In addition there is possibly a slight increase in color from 2.5 minutes to 4 minutes.

For accurate work, the colorimetric determination should be carried out within one or two hours after boiling, since there is a small increase in the intensity of the color during the first twenty-four hours as shown in Fig 4. After twenty-four hours there is a decrease in color. The initial value is obtained again in a couple of days.

The method has the advantage that the entire range of glucose concentrations usually encountered (0.2 to 10 per cent) can be determined without the necessity of making different dilutions of urine. This not only saves time but

also eliminates an important source of mistakes in computation. Using the Coleman Junior Spectrophotometer and 19 mm test tubes it was found that the whole range could be covered by employing 0.1 cc of urine to 10 cc of the reagent and determining the developed colors at two different wave lengths

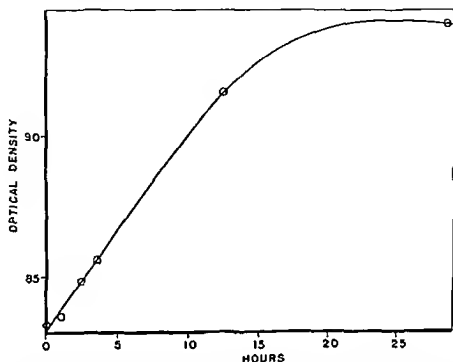


Fig 4—The density after boiling as a function of the time during which the tubes remained at room temperature. Each point is the average of five determinations.

For lower concentrations up to 0.6 per cent glucose 540  $m\mu$  was found to be optimal, whereas for concentrations in the range 0.6 to 10 per cent 700  $m\mu$  was found to be optimal. For other types of colorimeter or for this colorimeter with a different sized tube a different wave length may be found to be better. To avoid mistakes it is preferable always to record the densities of all samples at both of the two wave lengths.

It is ordinarily recommended that wave lengths be employed for which the substance to be determined has a maximum or minimum of absorption. This could not be done in this instance since no such maximum or minimum is present.

Using other types of colorimeters it may be necessary to dilute the reaction mixture. If so dilution with water should be avoided since the color is unstable except in strong solutions of sodium hydroxide. Dilution may be made with 5 per cent sodium hydroxide which does not influence the stability of the color. In this case the reagent blank used as a standard must be diluted in the same proportion. It is easier however to dilute with the reagent itself in which case the reagent blank can be used unchanged.

The colors of the solutions were found to be dependent to a considerable degree upon temperature. Thus the density of the reagent increased 20 per cent with an increase in temperature from 20° to 30° C. whereas the density of the strongly colored solutions resulting from high sugar concentrations increases only 9 per cent with the same change in temperature. Therefore it is necessary first, to keep the reagent blank and the reaction mixture at the same temperature and, second, to take this temperature into consideration in the calculation. To be safe, it is always best to use one reagent blank with

TABLE III THE GLUCOSE CONCENTRATION AS A FUNCTION OF THE OPTICAL DENSITY  
 Along With the Probable Value of the Concentration Are Given Upper and  
 Lower Limits, Calculated in Such a Way That the Probability Is About  
 99 Per Cent That the True Value Is to Be Found Within Them

TEMPERATURE 22° C							
% GLUCOSE OR EQUIVALENT AMT OF OTHER REDUCING SUBSTANCE				% GLUCOSE OR EQUIVALENT AMT OF OTHER REDUCING SUBSTANCE			
DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT	DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT
540 Mμ							
01	11	00	20	52	36	33	39
02	12	00	21	54	36	33	39
03	13	05	21	56	37	34	40
04	13	07	21	58	38	35	41
05	14	08	21	60	39	36	42
06	14	09	22	62	40	37	43
07	15	10	22	64	41	38	44
08	16	10	22	66	42	39	45
09	16	11	22	68	42	39	45
10	17	12	23	70	43	40	46
12	18	13	23	72	44	41	47
14	19	15	24	74	45	42	48
16	20	16	24	76	46	43	50
18	21	17	25	78	47	44	51
20	21	18	25	80	48	45	52
22	22	19	26	82	49	45	53
24	23	20	26	84	50	46	54
26	24	21	27	86	51	47	55
28	25	22	28	88	53	49	57
30	26	23	29	90	54	50	58
32	27	24	30	92	55	51	59
34	28	25	31	94	57	53	61
36	29	26	32	96	58	54	62
38	30	27	33	98	59	55	63
40	30	27	33	1 00	60	56	64
42	31	29	34				
44	32	29	35				
46	33	30	36				
48	34	31	37				
50	35	32	38				
700 Mμ							
06	50	40	60	26	22	20	24
07	60	50	70	27	23	21	25
08	65	55	75	28	24	22	26
09	75	65	85	29	25	23	27
10	80	70	90	30	26	24	28
11	90	80	1 00	32	28	26	30
12	95	85	1 05	34	31	28	34
13	1 05	95	1 15	36	33	30	36
14	1 15	1 00	1 30	38	35	32	38
15	1 20	1 05	1 35	40	37	34	40
16	1 30	1 15	1 45	42	39	36	42
17	1 40	1 25	1 55	44	42	38	46
18	1 50	1 35	1 65	46	44	40	48
19	1 60	1 45	1 75	48	47	43	51
20	1 65	1 50	1 80	50	49	45	53
21	1 70	1 55	1 85	52	52	48	56
22	1 80	1 65	1 95	54	54	50	58
23	1 90	1 75	2 05	56	57	52	62
24	2 00	1 85	2 15	58	59	54	64
25	2 10	1 90	2 30	60	62	57	67

TABLE III—CONT'D

TEMPERATURE 22° C							
% GLUCOSE OR EQUIVALENT AMT OF OTHER REDUCING SUBSTANCE				% GLUCOSE OR EQUIVALENT AMT OF OTHER REDUCING SUBSTANCE			
DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT	DENSITY	PROBABLE VALUE	LOWER LIMIT	UPPER LIMIT
60	6.1	5.9	6.9	82	9.2	8.5	9.9
64	6.7	6.2	7.2	84	9.5	8.7	10.3
66	6.9	6.4	7.4	86	9.9	9.2	10.6
68	7.2	6.7	7.7				
70	7.4	6.9	7.9				
72	7.7	7.2	8.2				
74	8.0	7.4	8.6				
76	8.2	7.7	9.0				
78	8.6	8.0	9.2				
80	8.9	8.3	9				

each lot of tubes boiled. In this way one can be sure that there is no great temperature difference between reagent blank and reaction mixture. Table III is calculated for a temperature of 22° C. For other temperatures a correction\* must be made. Table IV shows the values for this correction. These are applicable, no matter what type of colorimeter is used.

TABLE IV. TEMPERATURE CORRECTIONS

GLUCOSE CONCENTRATION FOUND FROM TABLE III (%)	TEMPERATURE (°C)				
	15	20	25	30	35
	CORRECTION (% GLUCOSE)				
1	00	00	00	00	01
2	01	00	00	01	02
3	02	00	00	02	03
4	03	01	01	02	04
5	03	01	01	03	05
6	03	01	01	03	06
8	04	01	01	04	08
10	05	02	02	05	10
15	08	02	03	08	15
20	10	03	04	10	20
25	13	04	06	13	25
30	1	05	08	15	30
40	20	06	00	20	40
50	25	08	12	25	50
60	3	1	1	3	6
70	4	1	1	4	7
80	4	1	2	4	8
90	5	1	2	5	9
100	5	2	2	5	10

For computation of the sugar concentration from the density a set of two curves can be used, one for each of the two wave lengths. For routine use, however, a table such as Table III is more convenient and is less subject to mistakes in application.

It should be pointed out that the relation between density and glucose concentration is not linear. It is especially remarkable that small amounts of glucose give no color at all. Thus a density reading of approximately zero means

\*For clinical work in which approximate values are satisfactory and in which laboratory temperatures vary only a few degrees this correction is unnecessary.

not that there is no glucose in the urine but that there is less than about 0.1 per cent. For clinical purposes glucose concentrations as low as this are usually of no significance.

For the purpose of rough estimations, where an error of  $\pm 20$  per cent is unimportant, the standard curve may be determined by applying the previously described procedure to known solutions of glucose. If limits of error of about  $\pm 10$  per cent are required, great care must be taken to make the standard curve exact enough. In this work the following procedure was employed.

The glucose content of a commercial dextrose preparation was determined by polarimetry. From this preparation a solution was made up, containing a known amount of glucose per gram. A portion of this solution was weighed out, water added to make a total volume of 1 c.c., and 100 c.c. of reagent were added. After thorough mixing, the solution was divided into ten portions in each of ten test tubes and heated in boiling water for three minutes. After cooling, the color was determined in the usual manner using ten different tubes of reagent blank. The average of the ten readings makes up one point of the curve. The figures in Table III are read from this standard curve.

A number of calibrated colorimeter tubes were used for determination of the density of the same solution. They were found to vary slightly, the standard deviation being  $\pm 0.5$  per cent. Other colorimeter errors were found to cause a standard deviation of  $\pm 1$  per cent. The errors in determining the points on the standard curve, made in the previously described manner, were found to cause a standard deviation in the apparent glucose concentration of  $\pm 0.4$  per cent for values higher than 0.3 per cent glucose.

The upper and lower limits of error, given in Table III, were calculated from these figures together with the previously mentioned pipette errors. These limits are determined in such a way that the probability is about 99 per cent that the true value is to be found within them.

The method described is sufficiently accurate for clinical work. The Benedict titration, in the form in which it is carried out in many clinical laboratories that is, without any precautions to prevent the oxidizing influence of the air, is much less accurate. Titrations of eleven portions of a single sample of urine from a diabetic patient showed the following percentages of glucose: 13, 14, 0.9, 1.3, 1.2, 0.8, 1.0, 1.1, 1.9, 1.9. The proportion between the highest and the lowest of these values is more than 2:1.

*Interference by Substances Other Than Glucose*—In the present work, it was assumed that no sugars other than glucose were present. The fact that different sugars give different reduction rates with the dimethylglyoxime reagent has been utilized by Epton<sup>6</sup> for the identification of these substances in urines. We have found, however, that the relative rates of reduction depend not alone upon which sugar is present but also to some extent upon the concentration. Thus, further investigations must be carried out before the method can be adapted to routine use for the differentiation of melliturias.

Other reducing substances present in urine, such as creatinine, uric acid, phenols, and amino acids, may interfere with the determination of glucose by



most of the methods in common use. In order to test their effect on the present method, the following substances were added to the reagent in amounts corresponding to very high concentrations in the urine: 11 per cent phenol, 22 per cent salicylic acid, 10 per cent acetone, 30 per cent urea, 10 per cent oxalic acid and 3 per cent uric acid. None of these substances gave any measurable color. The color produced by creatinine has been mentioned previously, 0.16 per cent creatinine in the urine was found to give a density of 0.03 after standing at room temperature for an hour. For most clinical purposes reduction by creatinine can be neglected.

A number of urines from dogs were determined both by this method and by the Benedict titration (Table V). In about half of the "normal" urines the Benedict method yielded considerable amounts of reducing substances, the maximum being 0.9 per cent expressed as glucose equivalent and values of about 0.6 per cent being very common. In one such urine no fermentation occurred with yeast, whereas a nonreducing urine to which was added 0.6 per cent glucose gave a slight fermentation. In contrast by the dinitrosalicylic acid method much lower reducing values were found in the same urines, usually about half as much as by the Benedict titration, indicating a higher degree of specificity of the present method.

TABLE V REDUCING POWER OF DIFFERENT SAMPLES OF DOG URINES AS DETERMINED BY THE BENEDICT METHOD AND BY THE DINITROSALICYLIC ACID METHOD  
Values Are Expressed as Per Cent Glucose Equivalent

BENEDICT	DINITROSALICYLIC ACID
0.1	Less than 0.1
0.25	0.1
0.7	0.26
0.33	0.15
0.25	0.1
0.6	0.27
0.6	0.34
0.83	0.44
0.6	0.33
Less than 0.1	Less than 0.1
0.4	0.14
0.30	0.12
0.58	0.36
0.56	0.30
0.40	0.36
0.51	0.23

#### SUMMARY

A modification of Sumner's method has proved very satisfactory as a routine method for the determination of glucose in urine. The procedure has the advantage that the entire range of glucose concentrations from 0.2 to 10 per cent is covered without using different dilutions of the urine. The limits of error are within  $\pm 10$  per cent for concentrations higher than 0.3 per cent glucose. For such concentrations the influence of nonsugar reducing substances can be largely neglected. This method is thus more specific than other routine methods. If sugars other than glucose are present, however, erroneous results will be obtained.

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# A SIMPLE METHOD FOR DETERMINING SULFONAMIDE SENSITIVITY IN VITRO AND ITS CLINICAL APPLICATION

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THIS communication describes a simple method for the determination of the relative sensitivity of a given bacterial strain to different sulfonamides. A method for this purpose has not heretofore come into clinical use in part because various substances contained in the usual culture media, particularly peptone,<sup>1</sup> prevent a determination of the absolute antibacterial effect of sulfonamides. Special media which do not contain such inhibitors have been prepared,<sup>2</sup> but the techniques involved are too laborious for practical clinical application. Peptone containing media however, if properly prepared, can be used for determining the relative antibacterial potency of various sulfonamides against a given bacterial strain. The method described below enables one not only to determine the sensitivity of a given bacterial strain to a series of sulfonamides but also to choose the most potent one.

## METHOD

Nutrient broth (Difco) containing 0.5 per cent peptone was adjusted to a pH of 8.2 in order to obtain maximum solubility of the sulfonamides. The amount prepared was sufficient to serve for all procedures of the entire study. For growth of the more fastidious bacteria the pH was lowered (pH 7.6) and horse serum (5 per cent) and glucose (1 per cent) were added. Serial dilutions of each of a 1,000 mg. per cent stock solution of the various sulfonamides were prepared in concentrations of 750, 500, 200, 100, 50, 25, and 10 mg. per cent respectively. One cubic centimeter of each of the eight dilutions was placed in Wassermann tubes. To each tube was added 0.1 cc. of a bacterial suspension prepared by diluting an eighteen to twenty-four hour culture so that 0.1 cc. contained 1,000 to 20,000 bacteria. A control tube containing broth and no sulfonamide was inoculated with 0.1 cc. of the same bacterial suspension.

Such small inocula were used because for some strains large inocula are said to depress the activity of the sulfonamides.<sup>3</sup> The broth prepared as described was used to prepare all stock solutions, all the dilutions of the sulfonamides, and the bacterial cultures and dilutions. The tubes were incubated for twenty-four hours at 37° C. The lowest concentration of each sulfonamide which completely inhibited macroscopically visible growth was taken to be the bactericidal titer. The concentration of each sulfonamide which produced a recognizably lesser degree of turbidity than the control tube was considered to be the bacteriostatic titer.

We are aware of the fact that this method of reading an *in vitro* test is somewhat inaccurate. The last clear tube may contain a small number of viable bacteria, which may be destroyed within another twenty-four hours or may start to multiply after twenty-four hours have elapsed. Also a tube macroscopically just as cloudy as the control tube might well contain fewer organisms than the latter. However for practical purposes these possibilities can be neglected, as will be shown in the discussion of the results.

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Acknowledgment is due Miss Sunya Gordon and Miss Annette Freedman for technical assistance.

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## RESULTS

Two hundred bacterial strains were studied. Markedly different action was observed by various sulfonamides on many strains of *Escherichia coli*, *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Eberthella typhi*, *Salmonella schottmulleri*, *Salmonella enteritidis*, *Bacillus proteus vulgaris*, and *Pseudomonas aeruginosa* and among some strains of *Staphylococcus aureus hemolyticus*, but only rarely on various strains of hemolytic streptococci and pneumococci. Some bacterial strains of each of these species were equally resistant while others were equally sensitive to all sulfonamides studied. Gram-negative cocci and gram positive bacilli were not examined. Table I provides a few significant examples.

## CLINICAL OBSERVATIONS

The clinical importance of such tests for selecting the most effective sulfonamide is emphasized by the following representative case histories.

CASE 1—A 25 year old man with pneumonia of the right upper lobe was treated with large doses of sulfadiazine for three days without effect. At this time, the process extended to the right middle lobe. A pneumococcus Type 1, isolated from blood and sputum, was completely resistant in vitro to penicillin, sulfadiazine, sulfamerazine, and sulfamethazine. The bactericidal titer with sulfathiazole was 100 mg per cent. Administration of sulfathiazole in adequate dosage led to recovery within thirty six hours.

CASE 2—A sequestrectomy was performed in a girl of 10 with osteomyelitis of the right tibia of four to five weeks' duration. Penicillin and sulfadiazine were administered without effect. Temperature remained high. A hemolytic, coagulase positive *Staph aureus*, isolated from the draining sinus in pure culture, was completely resistant in vitro to penicillin, sulfadiazine, sulfamerazine, and sulfamethazine. The bactericidal titer with sulfathiazole was 250 mg per cent. Following sulfathiazole therapy, fever rapidly declined, drainage subsided, and the patient recovered.

CASE 3—A 70 year old woman with acute postoperative cystitis due to *Esch coli* was treated for five days with full doses of sulfadiazine without effect. The strain was completely resistant in vitro to sulfadiazine, sulfathiazole, sulfamethazine, and streptomycin. The bactericidal titer with Nu 445 (3,4 dimethyl 5 sulfanilamido isovazole) was 100 mg per cent. There was complete clinical and bacteriologic cure after three days of treatment with Nu 445.

CASE 4—A 40 year old woman with acute pyelonephritis and cystitis due to *Esch coli* was treated for six days with full doses of sulfadiazine without improvement. The bactericidal titer with sulfadiazine, sulfathiazole, and sulfamethazine was 500 mg per cent, but with Nu 445 it was 250 mg per cent. Clinical and bacteriologic cure was achieved within six days with Nu 445.

CASE 5—Following prostatectomy a 59 year old man developed acute cystitis and bilateral pyelonephritis due to *A. aerogenes* and *Ps. aeruginosa*. Prolonged therapy with sulfadiazine and later with sulfamerazine was ineffective. Both strains were resistant in vitro to sulfadiazine, sulfamerazine, and sulfathiazole. The bactericidal titer of the *A. aerogenes* with sulfamethazine was 100 mg per cent, of the *Ps. aeruginosa*, 250 mg per cent. Treatment with this drug rapidly cured the infection.

CASE 6—A 66 year old woman following perineorrhaphy developed acute cystitis due to *Ps. aeruginosa* and *B. faecalis alcaligenes*. Penicillin in large doses was without effect. In vitro tests showed a bactericidal titer for the *B. alcaligenes* of 100 mg per cent with sulfadiazine, sulfathiazole, and sulfamethazine. The *Ps. aeruginosa* was completely resistant to

TABLE I COMPARATIVE IN VITRO SENSITIVITY OF SEVERAL STRAINS OF A GIVEN SPECIES OF BACTERIA TO VARIOUS SULFONAMIDES (SIZE OF INOCULUM, 2,000 TO 8,000 BACTERIA, TITERS IN MG % OF DRUG IN NUTRIENT BROTH)

STRAIN	SD		SM		SMT		ST		PST		Nu 415	
	c	st	c	st	c	st	c	st	c	st	c	st
Fsch coli 1	300	300	250	50	25	25	500	100	750	250	750	500
Fsch coli 2	300	100	250	100	250	100	100	50	500	250	1000	500
Fsch coli 3	1000	200	400	250	750	250	500	250	750	500	100	25
Fsch coli 4	1000	750	1000	1000	500	100	50	25	500	250	1000	1000
A aerogenes 1	>1000	1000	>1000	1000	100	100	500	250	500	250	>1000	>1000
A aerogenes 2	>1000	>1000	>1000	1000	750	500	250	250	750	500	>1000	>1000
A aerogenes 3	750	750	750	750	500	250	100	50	750	250	>1000	>1000
A aerogenes 4	1000	750	>1000	>1000	250	100	400	250	750	750	>1000	>1000
B proteus 1	200	25	300	500	100	50	750	50	750	250	1000	25
B proteus 2	750	750	1000	750	500	100	500	250	500	250	100	100
B proteus 3	>1000	>1000	>1000	750	500	250	100	25	500	250	1000	25
B proteus 4	300	200	300	250	500	250	750	250	750	250	750	750
Is aeruginosa 1	>1000	>1000	>1000	>1000	500	250	>1000	>1000	>1000	>1000	1000	750
Is aeruginosa 2	>1000	1000	>1000	>1000	1000	750	200	100	500	250	>1000	>1000
Is aeruginosa 3	1000	1000	1000	750	750	500	500	250	750	750	>1000	>1000
Is aeruginosa 4	1000	1000	>1000	>1000	1000	300	750	750	750	750	100	50
Staph aureus 1	500	50	200	100	500	300	1000	750	>1000	1000	1000	1000
Staph aureus 2	1000	750	>1000	1000	100	50	200	250	750	750	1000	1000
Staph aureus 3	100	100	200	250	500	750	750	500	750	750	>1000	>1000
Staph aureus 4	700	500	700	500	500	250	100	20	500	750	>1000	>1000

SD Sulfadiazine SM Sulfamerazine SMT Sulfamethazone ST Sulfathiazole PST Sulfathalidine Nu 41 3 4 dimethyl 5 sulfamylamido oxazole

SD Sulfadiazine SM Sulfamerazine SMT Sulfamethazine ST Sulfathiazole PST Sulfathidine Nu 44 3,4 dimethyl 5 sulfamido isoxazole

c Bactericidal titer at bacteriostatic titer

All strains were freshly isolated from human infections Only those showing marked difference in sensitivity against various sulfonamides are listed

Titers of 50 mg per cent or less are likely to be therapeutically effective

sulfadiazine and sulfathiazole The bactericidal titer with sulfamethazine was 100 mg per cent Sulfamethazine administration led to complete clinical and bacteriologic cure within three days

### DISCUSSION

The method described is based on the assumption that the inhibitors contained in the broth do not affect the relative potency of the sulfonamides used in these tests, even if they do substantially lower the absolute potency From the available evidence<sup>3, 7, 8</sup> it appears that the same amount of inhibitor affects sulfadiazine, sulfathiazole, and sulfapyridine equally While such data are not available for sulfamethazine or Nu 445, our experience suggests that this is probably also the case for them

Although the action of a sulfonamide on a given strain is stronger the simpler the composition of the medium,<sup>3, 7</sup> we observed that the marked differences in relative potency of the various sulfonamides on a given bacterial strain were exactly the same whether a simple synthetic medium or a broth was used (Table II) The difference in potency is of the order of one to two tubes This observation indicates that the foregoing method is sufficiently accurate for routine laboratory work

TABLE II COMPARISON OF BACTERICIDAL TITERS IN MG PER CENT OF STRAINS OF ESCH COLI IN NUTRIENT BROTH AND IN THE SYNTHETIC MEDIUM OF KOHN AND HARPIS<sup>7</sup> IN VARIOUS SULFONAMIDES

STRAIN	NUMBER OF BACTERIA	MEDIUM (pH 7.2)	SD	SM	SMT	ST	PST	Nu 445
E coli 1	2,000	Broth	750	750	250	100	250	500
		Synthetic med	250	250	50	25	50	100
E coli 2	4,000	Broth	1,000	750	500	100	500	750
		Synthetic med	750	500	250	50	250	500
E coli 3	3,000	Broth	1,000	1,000	750	1,000	1,000	250
		Synthetic med	750	750	500	750	750	100
E coli 4	4,000	Broth	500	500	100	250	500	750
		Synthetic med	250	250	50	100	250	500

SD sulfadiazine SM sulfamerazine SMT sulfamethazine ST sulfathiazole, PST sulfathalidine Nu 445 3,4-dimethyl-5 sulfanilamido-isoxazole

Similar comparative tests done with strains of *A. aerogenes*, *B. proteus vulgaris* and *P. aeruginosa* gave similar results

From the close correlation between results of the in vitro test and the therapeutic effect, two further conclusions may be drawn (1) The choice of the sulfonamide should be made on the basis of the bactericidal and not the bacteriostatic titer The gap between the two titers varies with nearly every strain While they are occasionally identical, the bacteriostatic titer is often from one tube to three to four tubes removed from the bactericidal titer (2) A sulfonamide exerting bactericidal action on a given strain in a concentration of 250 mg per cent or lower is very likely to be therapeutically effective If the bactericidal titer is 500 mg per cent, the clinical action of the drug is questionable but the drug should be tried if a more effective agent is not available Sulfonamides with a bactericidal titer of 750 mg per cent or higher are not therapeutically effective

This study emphasizes the fact\* that the sensitivity of different strains of a given species of bacteria is extremely variable. Some strains may be very sensitive and others completely resistant to the same sulfonamide.

## SUMMARY

A simple method for determining the *in vitro* effect of various sulfonamides on a given bacterial strain is described. Clinical experiences are given showing the importance of the correct choice of sulfonamide.

The fact that a majority of the strains of a given species are usually sensitive to a sulfonamide does not assure a therapeutic effect in a given instance. Hence it is frequently necessary to determine the relative sensitivity of the strain responsible for the infection to a series of sulfonamides.

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# A MODIFIED ULTRAVIOLET SPECTROPHOTOMETRIC METHOD FOR QUANTITATIVE DETERMINATION OF BARBITURATES

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## INTRODUCTION

THE three drug depressions most commonly encountered in clinical medicine are due to the excessive absorption of alcohol, bromides, or barbiturates.

The first two of these agents may be determined readily, both qualitatively and quantitatively, by well-established clinical laboratory techniques. The qualitative detection of barbiturates also can be carried out with a fair degree of success by the Koppanyi method providing sufficient blood or urine samples are available. However, until recently, quantification of the barbiturate levels in sera was not feasible since large quantities of blood were necessary.

Recently, spectrophotometric techniques for quantitative barbiturate determinations in sera have been proposed by others.<sup>1,2</sup> These techniques are extremely sensitive, so that an accurate barbiturate determination can be made on as little as 1 ml of blood. The method developed in this laboratory has a distinct advantage over previous methods in that it can be applied directly to serum extracts with the elimination of interference due to serum and reagent blanks. We have used this procedure as a diagnostic aid in over fifty cases of barbiturate intoxication during the past year and have followed the blood levels of a number of these patients during their course of recovery in the hospital.

Determination of barbiturate sera levels aids the clinician in evaluating the relative effectiveness of various therapeutic measures he employs in his treatment of barbiturate intoxication.

## EXPERIMENTAL

*General Description of the Method*—The barbiturate is extracted directly from serum in a semimicro continuous extractor using diethyl ether. The ether is evaporated and the barbiturate residue is brought into solution as the sodium salt using a borate buffer of pH 9.5. Optical density of the solution is determined by means of a Beckman spectrophotometer, and the amount of drug present is calculated using a standard curve prepared by a method which eliminates blank serum interference.

*Importance of the Influence of pH on the Absorption Spectra*—The influence of pH on the absorption spectra of barbital and the 5,5-disubstituted barbiturates was reported by Elvidge,<sup>3</sup> Stuckey,<sup>4</sup> and more recently by Walker and co-workers.<sup>5</sup> We have confirmed these studies by measuring the absorption curves of six barbiturates in aqueous solution at various pH values between 7.0 and 12.0. The barbiturates studied were Amytal, barbital, cyclobarbital, Cyclopal, pentobarbital, and phenobarbital. The absorption curves for amytal, which are similar to those of the other barbiturates tested, are shown in Fig. 1.

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The observation, as reported by Goldbaum that each barbiturate gives a characteristic absorption curve at pH 11 or greater suggested the possibility that the ratios of the optical densities at specific wave lengths could be used as criteria for the differentiation of the various barbiturate derivatives. The use of this means of identification is of little practical significance in clinical analysis however since the marked absorption due to blood or serum

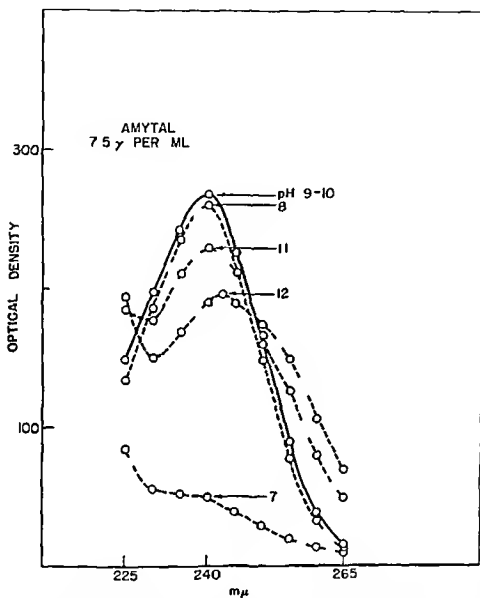


Fig 1—Absorption curves of Amytal in aqueous solution at varying pH values

usually obscures not only the characteristic maximum but the other critical portions of the curve. The greatest amount of absorption per unit concentration is obtained between the pH values 9.0 and 10.0. Within this range of alkalinity all 5,5-disubstituted barbiturates develop peak absorption in the wave length range from 239 to 240 millimicrons. Although it is not possible to differentiate any one of the barbiturates by the shape of its absorption spectra in this pH range, the type of curve obtained with its steep slope is least likely to be obliterated by interfering serum blanks.

Thus the optimum pH range for measurement of the absorption spectra of barbiturates was found to be between 9.0 and 10.0. In the determination of drug levels in serum unless the solution is carefully maintained in this range of alkalinity variations may appear in the absorption curve which give erratic results. With reference to Fig. 1, it is evident that measurement of optical density at a pH of 11, rather than from 9 to 10 would result in only about 85 per cent recovery. For this reason we have used a borate buffer of pH 9.5 as the solvent in the determination of barbiturate absorption.

**Interference by Serum Blanks**—Interfering absorption by ether or chloroform extracts of serum proved to be a problem of considerable difficulty. In experimental work

a blank serum specimen can be determined prior to the administration of the drug, and, provided no change occurs in the blank, a correction factor may be applied for the subsequent barbiturate determination. In clinical and forensic medicine, however, these blank samples are rarely available. Thus, the amount of absorption due to serum itself is unknown, and the accuracy of the quantitative determination of the drug is subject to a variable degree of error.

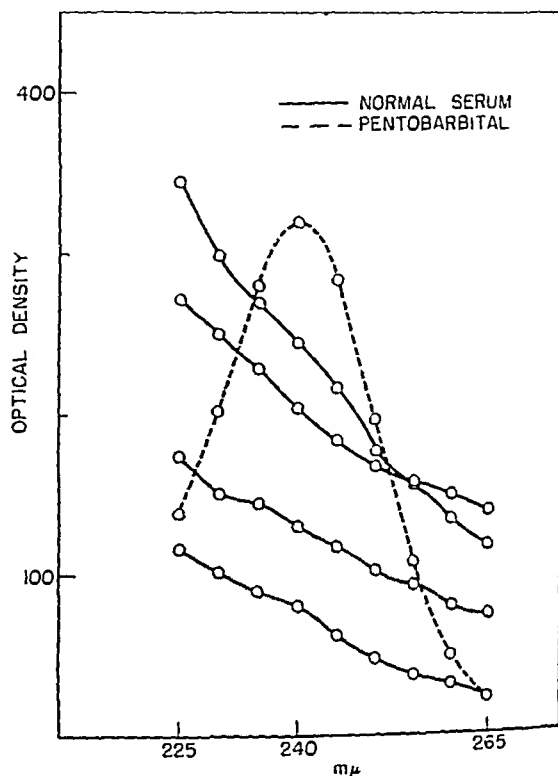


Fig 2—Absorption curves of ether extracts of normal human serum in borate buffer (pH 9.5). The absorption curve of pentobarbital (8γ per milliliter in borate buffer of pH 9.5) is superimposed to illustrate the importance of serum interference.

Goldbaum<sup>2</sup> has suggested an optical density value of 0.030 to be applied as a control blank in toxicologic analyses where no barbiturate-free samples of normal blood are available. This value was reported as the average for chloroform extracts of all normal blood samples measured at 255  $m\mu$  in 0.5N sodium hydroxide. Determinations were made in this laboratory of the optical densities of both ether and chloroform extracts of a series of 150 sera obtained from patients having had no history of recent drug ingestion. The optical densities of these "normal" sera were found to vary between 0.040 and 0.250 at wave length 240 millimicrons. Serum absorption measured at 255  $m\mu$  was only slightly less. The absorption curves of a few typical specimens of normal human serum are shown in Fig 2, with an absorption curve of pentobarbital superimposed to illustrate the importance of blank serum interference. It is evident that no average blank value of any significance can be derived from these results.

Walker and associates have reported a method for the elimination of blank interference due to blood in which they employ an acid-alkaline shift.<sup>1</sup> The procedure is not applicable to direct extracts of blood or serum, as stated by Walker and confirmed by our experiments, and must be carried out on the filtrate of a tungstate-sulfuric acid protein precipitation. In so doing approximately 25 to 35 per cent of the barbiturate is unaccounted for, a factor which limits the accuracy of the method.

A satisfactory method of correcting for the interference of absorption due to serum and reagent blanks was developed in this laboratory by adapting a procedure described by Tunncliffe. The correction can be applied to extracts made directly from serum without preliminary protein precipitation. Fig 3 illustrates an actual problem demonstrating that the curve obtained from any clinical specimen (pentobarbital in serum)

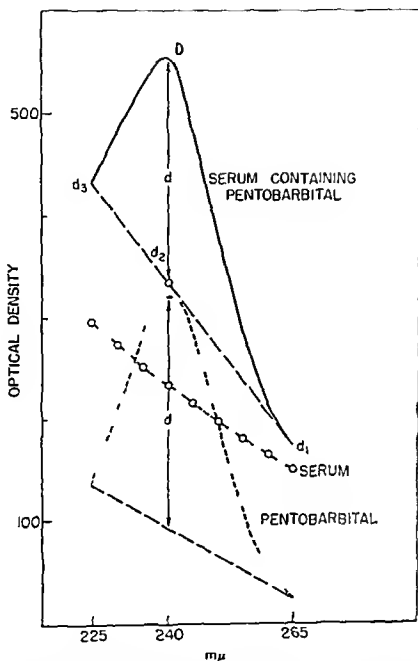


Fig 3—Absorption curves of ether extracts of serum and pentobarbital in serum and standard pentobarbital curve. All absorption curves were determined in borate buffer at pH 9. The pentobarbital concentration was 8γ per milliliter.

is the additive effect of the blank serum curve plus the actual barbiturate curve. It is evident that the peak absorption D cannot be used as a measurement of drug concentration since an unknown fraction of its magnitude is due to serum absorption. The method we have adopted is based on the observation that the absorption curves of normal blank sera conform approximately to a straight line in the range of wave lengths from 225 to 265 mμ (Fig 2). Thus with reference to Fig 3 if a base line is drawn between the optical density values  $d_3$  and  $d_1$  a value  $d$  is obtained which serves as an adequate index for the amount of barbiturate present since it is directly proportional to drug concentration, and it is not significantly altered by serum blanks. This  $d$  value may be obtained either graphically

by plotting the three values,  $d_3$ ,  $D$ , and  $d_1$ , and drawing the base line  $d_3d_1$ , or by simple algebraic calculation using the equations

$$d = D - d_1 \quad (A)$$

$$\text{where } d_1 = \frac{5}{8}d_3 + \frac{3}{8}d_1 \quad (B)$$

The derivation of (B) is as follows

$$\frac{d_3 - d_1}{d - d_1} = \frac{\lambda_3 - \lambda_1}{\lambda_2 - \lambda_1} \quad (C)$$

$$d = d_3 \frac{(\lambda - \lambda_1)}{(\lambda_3 - \lambda_1)} - \frac{d_1 (\lambda - \lambda_3)}{(\lambda_3 - \lambda_1)} \quad (D)$$

Substituting wave length values in Equation (D) and solving

$$d_1 = d_3 \frac{(240 - 265)}{(225 - 265)} - \frac{d_1 (240 - 225)}{(225 - 265)} \quad (E)$$

which results in (B)

$$d = \frac{5}{8}d_3 + \frac{3}{8}d_1 \quad (B)$$

A practical example will be cited to illustrate how the calculation may be applied (Table I). Following the regular extraction procedure the optical density was determined on I, a blank sample of human serum, and III, the same serum to which had been added a known quantity of pentobarbital. II represents the standard absorption curve of the concentration of pentobarbital present in the serum. Table I includes the data as calculated algebraically using Equations (A) and (B). Fig. 3 illustrates the example graphically.

TABLE I ILLUSTRATION OF ACCURACY OF CALCULATION OF BARBITURATE CONCENTRATION BY THE BASE LINE METHOD

WAVE LENGTH	SYMBOL	OPTICAL DENSITY		
		BLANK SERUM I	PENTOBARBITAL II	PENTOBARBITAL IN SERUM III
225 mμ	$d_3$	0.295	0.137	0.432
240 mμ	$D$	0.235	0.320	0.555
265 mμ	$d_1$	0.150	0.023	0.173
	$d_2$	—	0.094	0.335
	$d$	—	0.226	0.220
/ per ml		—	5.0	7.8

By the use of this method the interference due to serum blanks is almost completely eliminated. In the foregoing example, the calculated value of barbiturate level is 97.5 per cent of the actual level. In practice, the accuracy of the method depends in part upon the nature of the blank serum absorption curve. The curve as a whole, from 225 to 265 mμ, does not always coincide exactly with the base line  $d_3d_1$ . This is not of great importance, however, since it is the amount of deviation in optical density between the serum curve and the base line at 240 mμ which is significant. This deviation is rarely more than shown in the example given, 0.006 optical density. The dependability of this correction for blank interference is reflected in the data contained in Table II.

#### PROCEDURE

1 One milliliter of serum is placed in the extractor, and from 5 to 10 ml of distilled water are added, depending upon the volume of the extractor. Larger volumes of water are

be used when available. Approximately 30 ml of diethyl ether are placed in the receiving flask.\* The apparatus is assembled as shown in Fig 4 the ether being evaporated in a water bath regulated between 50 and 55 C. In the event that emulsions are formed at the interface, they are readily removed by addition of a few crystals of anhydrous sodium sulfate. When

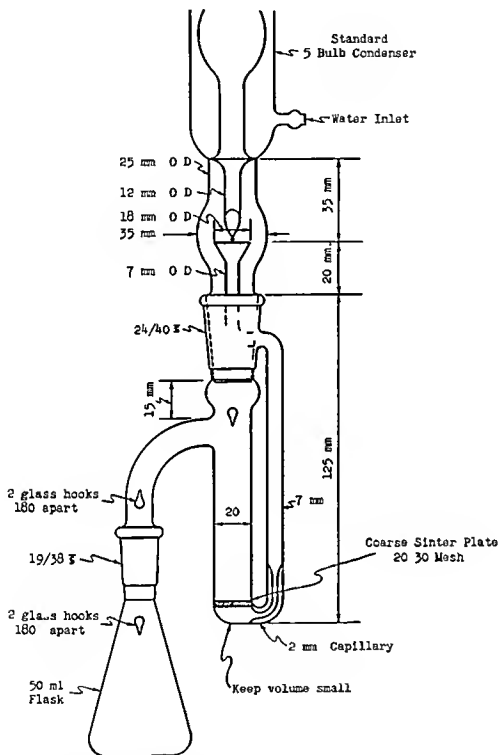


Fig 4—Diagram of semimicro extraction apparatus

extraction is complete (fifteen to sixty minutes) the ether may be evaporated into a clean extractor and saved for redistillation. The receiving flasks containing the barbiturate residue must be completely dry before addition of the borate buffer. This is best accomplished by placing them in a drying oven for a few minutes.

**Reagents** Diethyl ether technical grade. Washed with distilled water, treated with ferrous sulfate, dried with anhydrous calcium chloride and redistilled, preferably over metallic sodium.

**Borate buffer** 0.2M boric acid C.P.  
0.1M potassium chloride C.I.—Solution A  
0.2M sodium hydroxide C.P.—Solution B

prepared according to Peters and Van Slyke to pH 9.  
**Apparatus** Semimicro continuous extractor with condenser and receiving flask (Fig 4).  
Water bath with thermoelectric control ranging from 40 to 55 C. Beckman Model DU Ultra violet spectrophotometer with 1 cm quartz absorption cell.

TABLE II RECOVERIES OF BARBITURATES ADDED TO BLANK SERUM  
(LEVELS WERE CALCULATED BY THE BASE LINE METHOD)

NUMBER OF DETERMINATIONS	MICROGRAMS ADDED	AVERAGE RECOVERY* (PER CENT)
11	14.4	92.2 ± 2.75
14	20.0	98.5 ± 1.95
12	75.0	101.2 ± 1.28
10	100.0	95.7 ± 1.81

\*This includes one standard error of the mean

2 When dry, the barbiturate residue is brought into solution with an accurately measured quantity of borate buffer (pH 9.5). A volume of 2 ml or more may be used, depending upon the concentration of drug present.

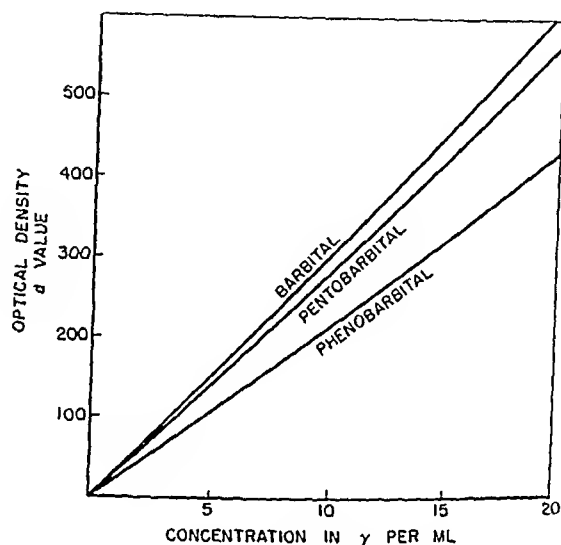


Fig 5—Standard concentration curves of a few representative barbiturates. The optical density is measured in *d* units calculated from Equations A and B.

3 With the barbiturate present in the alkaline form in the pH range from 9.0 to 10.0, absorption is determined at three wave lengths, 225, 240, and 265  $m\mu$  ( $d$ ,  $D$ , and  $d$ , respectively), and the  $d$  value is calculated by using Equations (A) and (B).

4 Barbiturate concentration in the borate buffer is determined by reference to a standard curve (Fig 5).

The value may be expressed as milligrams per cent in the serum according to the equation

$$\text{Mg per cent of barbiturate} = \frac{\gamma B}{10 S}$$

where  $\gamma$  = micrograms of barbiturate read from the standard curve

$B$  = milliliters of borate buffer

$S$  = milliliters of sample

*Accuracy of the Method*—Table II includes data from a series of experiments to determine the accuracy of the method. Known amounts of barbiturates were added to normal sera (beef, horse, and human) of unknown optical density levels. The samples were determined according to the procedure outlined in the preceding section with 1 ml serum and in all instances the base line method was used to determine the drug level. Amytal, barbitol,

pentobarbital, phenobarbital and Seconal were the representative barbiturates included in the study. No significant difference between the recovery of the five barbiturates was noticed.

#### DISCUSSION

*Base Line Method of Calculation*—The three wave lengths, 225, 240, and 265  $m\mu$ , were empirically chosen since this combination of wave lengths resulted in the greatest  $d$  value per unit concentration. For the actual barbiturate curve (Fig 3), the farther removed the values  $d_2$  and  $d_1$  are from  $d$  the greater the  $d$  index. There are, however, certain limiting factors at wave lengths less than 225  $m\mu$  there is a sudden steep rise in serum absorption as barbiturate absorption falls off. Beyond 265  $m\mu$ , although serum absorption is gradually becoming less, the drug absorption also decreases. The wave length 240  $m\mu$  is the logical place for the  $D$  absorption since peak absorption occurs here.

*Interfering Substances*—There are three types of compounds of clinical importance which might cause interference in the barbiturate determination.

1 *Sulfonamides* Although alkaline solutions of sulfonamides exhibit absorption in the critical range of the barbiturate spectrum they may be almost completely eliminated when present in serum by the process of extraction. By carrying out the extraction procedure at the pH of serum, only negligible interference by sulfonamides was obtained with levels as high as 10 mg per cent in serum. The absorption curve of the small amount of sulfonamide which is extracted is such that at 240  $m\mu$  there is no deviation of the curve from the  $d_2d_1$  base line. The following sulfonamides were tested at concentrations of 10 mg per cent and were not found to interfere with the determination: sulfanilamide, sulfadiazine, sulfaguanidine, sulfapyridine, sulfamerazine, sulfathiazole.

2 *Salicylates* The salicylates give absorption curves with two maxima, one in the critical range of barbiturate absorption and the other beyond the limit where absorption of the non-thio type of barbiturate occurs. Salicylates can be identified easily by their characteristic absorption curves and consequently should not be mistaken for barbiturates. There is at present no satisfactory method of eliminating their interference.

3 *Products of Barbiturate Metabolism* In the metabolism of the nonsulfur containing barbiturates the splitting of the malonyl urea ring yields a compound which does not produce interfering absorption in the ultraviolet range. It is possible that alterations of the barbiturate other than breakdown to a ureide could yield metabolic products which would contribute to the absorption spectrum. Whether or not these compounds would be extracted by our method and whether or not their absorption curves could be differentiated from those of the parent compound are problems which are in need of further investigation.

In cases of barbiturate intoxication where the identity of the barbiturate is not known, there is an inherent error in all spectrophotometric methods, since barbiturates differ to some degree in their molecular extinction coefficients. From a practical standpoint this difference is not of great importance. It has been our practice when the ingested barbiturate was not

known or could not be qualitatively identified to express the sera level in terms of pentobarbital, since the  $d$  values for this compound are closest to the mean of the commonly encountered barbiturates

#### SUMMARY

A method for quantitative barbiturate analysis has been developed which has the following advantages

1 The interference due to absorption of reagent and serum blanks is eliminated by means of a graphic solution or a simple algebraic calculation

2 Careful regulation of pH in determining ultraviolet absorption increases the accuracy of the determination

3 Samples as small as 1 ml of serum may be extracted directly without preliminary protein precipitation

4 There is a considerable economy of glassware and reagents involved as well as ease of extraction

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## FAT DETERMINATION IN FECES USING MOJONNIER EXTRACTION FLASKS

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IN FAT balance studies the methods commonly employed for the determination of the fat in feces have not been particularly satisfactory. The procedure of drying the fecal specimens is cumbersome and time consuming and an error is introduced because during the drying and grinding process part of the feces sticks to the walls of the receptacles. Extraction of fat with the Soxhlet apparatus is difficult where the water pressure is not constant and the water is not sufficiently cold to condense the ether vapors. This latter feature is particularly significant in warm climates. Extraction methods employing wet feces are simple and efficient but it is difficult to avoid emulsification in extraction with alcohol and ethyl ether, hence centrifugation is often necessary.

### METHOD

An adaptation of the Rees-Gottlieb fat extraction method applied to wet feces and using Mojonnier extraction flasks was suggested by Hilda F. Wiese of the Department of Pediatrics.

The feces are washed with distilled water into a tared jar or a Waring Blender for mixing. Water is added to make the sample sufficiently fluid to be drawn into a pipette. The total weight of feces and water is taken. After thorough mixing either in the Waring Blender or with an electric stirrer which can be inserted directly into the weighed jar, 3 to 8 ml. aliquots are withdrawn and transferred directly into the bottom chamber of the weighed Mojonnier fat extraction flasks. The extraction flasks are weighed directly on an analytic balance. If the total fat excreted is to be reported on the dry basis separate aliquots are measured out for the determination of total solids.

For the extraction a few drops of concentrated HCl are added, then 12 to 15 ml. 95 per cent alcohol. The flasks are tightly stoppered and shaken for thirty seconds. Then 10 ml. ethyl ether are added. The flasks are stoppered and shaken vigorously for sixty seconds. This is followed with 25 ml. petroleum ether and the shaking is continued for sixty seconds. The flasks are then allowed to stand ten to fifteen minutes for complete separation of the ether layer. The Mojonnier fat extraction flasks are so constructed that the ether layer can be poured off directly into weighed containers for evaporation of the solvents without danger of contamination from the aqueous layer (See Fig. 1). The extraction procedure is repeated using 5 ml. 95 per cent alcohol, 15 ml. ethyl ether and 15 ml. petroleum ether. If the fat content of the feces is very high it may be necessary to make a fourth extraction. Just before the last ether layer is poured off sufficient water is added to fill the bottom chamber so the ether layer can be removed completely. The ether extracts are evaporated on the water bath, the flasks are dried in a desiccator and weighed. With the given proportions of alcohol and ether emulsions rarely occur.

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The Mojonnier fat extraction flasks are manufactured by Mojonnier Brothers Company,  
4601 West Ohio Street, Chicago 44, Ill.

However, if emulsification results, this can usually be broken by the addition of a small amount of alcohol. Also, it is sometimes desirable to re-extract the fat residues with petroleum ether alone. These can be filtered readily through fat free cotton inserted into a small funnel.

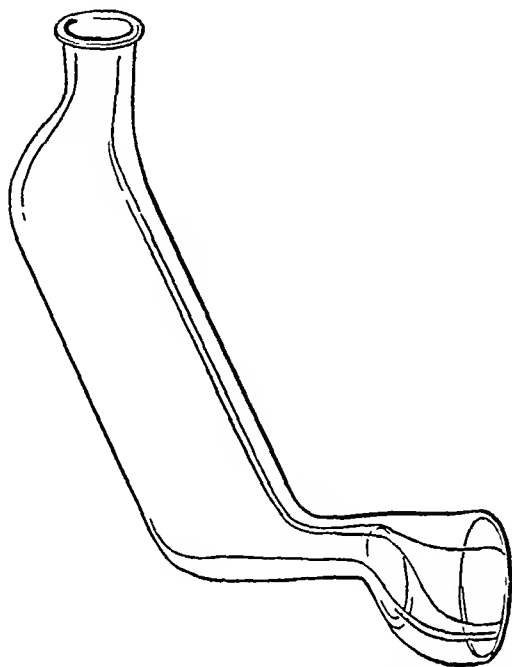


Fig. 1—Mojonnier flask used for fecal fat extraction

### RESULTS

In fifty consecutive duplicate determinations, forty-three showed a difference of less than 2.5 per cent. Two of the samples contained less than 1 per cent fat and the differences here were higher (3.3 and 6.1 per cent). These specimens were obtained from infants who were maintained on a skimmed milk mixture. In the other five instances, differences of 3.3 to 5.1 per cent were obtained. These latter determinations were made at the beginning of the study, and the maximum variation of 5 per cent in duplicate samples seemed satisfactory, hence they were not repeated.

The method described offers several advantages. There is little loss of feces in the preparation and transfer of the material. The extraction can be completed in a very short time and emulsification rarely occurs. Duplicate samples agreed within narrow limits, except in two instances where the fat content was less than 1 per cent of the sample.

## THE SIGNIFICANCE OF CHOLESTEROL VARIATIONS IN HUMAN BLOOD SERUM

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IT HAS been pointed out that the literature dealing with serum concentrations of lipids and cholesterol is filled with conflict<sup>1 2</sup> These inconsistencies appear to be accounted for by differences in technique and the unreliability of certain methods now widely used This subject has been reviewed adequately elsewhere<sup>3 4</sup> Accordingly, a modification of the Sperry Schoenheimer method<sup>4</sup> has been employed for cholesterol, as developed by Chaney and Lovell<sup>5</sup> This modification has been checked against the original Sperry Schoenheimer method and also against other procedures in wide use The results again emphasize the need for adhering to the fundamental principles of the Sperry Schoenheimer procedure and demonstrate that wide variations in results are likely to occur in other methods which do not adequately control the Liebermann Burchard color reaction The modification of Chaney and Lovell appreciably curtails the time required for the procedure and reduces the complexities of the original Sperry Schoenheimer method while maintaining its accuracy

The need for standardization and perfection of such technique has been even more emphasized by the recent renewal of interest in and investigations into the role of fat metabolism in the production of arteriosclerosis and in particular coronary thrombosis Recent studies suggested that serum cholesterol levels are subject to variations under certain conditions in the same subject at different time periods We therefore undertook an investigation into these circumstances of variation and present the following results

The additional value of such an inquiry would be in supplying further criteria on which to evaluate the role of certain lipotropic agents, such as choline, inositol, etc These have been recently employed both experimentally and clinically as "decholesterizing" agents in arteriosclerosis<sup>6 7</sup> Changes in serum cholesterol values were reported<sup>8</sup> following the use of these agents

### CLINICAL MATERIAL

Two or more fasting serum cholesterol tests were made at intervals in 161 subjects who were divided into four groups Group A consisted of thirty-two normal individuals who were free of any clinical symptoms or demonstrable evidence of any illness These subjects served as the normal controls Group B was selected as a control group comprising thirty-one patients who had various illnesses such as chronic peptic ulcer, chronic

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nephritis, chronic nonspecific ulcerative colitis, cirrhosis of the liver, chronic, rheumatoid arthritis, and essential hypertension. No patient in this group had known evidence of coronary occlusion or angina pectoris. Group C consisted of fifty patients who had had recent coronary occlusions (within a six month period of time) but who received no specific medication or lipotropic agents, except in a few instances where maintenance doses of digitalis or Mercuhydrin had been prescribed. Group D comprised forty-eight patients who had been on 6 Gm daily doses of choline bicarbonate for variable periods of time and who had suffered a recent coronary occlusion within six months of the institution of choline therapy. As in Group C, several patients were on maintenance doses of digitalis or Mercuhydrin.

#### RESULTS

*Group A*—Thirty-two normal subjects were studied whose ages ranged from 13 years to 72 years, the average age being 35 years. Twenty were male subjects, twelve were female. The time interval between the first cholesterol determination and the second (which was the last in most instances) ranged from eight weeks to sixty-four weeks, the average time interval being thirty-six weeks. As shown in Fig 1, the average initial serum cholesterol was 202 mg and the average final serum cholesterol was 200 milligrams. The variations between these two ranged from 0 to 38 mg, with an average variation of 11 milligrams. The normal serum cholesterol values are from 140 to 220 milligrams. Thus Fig 1 illustrates the constancy of cholesterol levels in normal controls, when two or more determinations separated by a considerable period of time (one to sixty-four weeks) are made on the same individual. It will be noted that the increase or decrease in level is slight, less than 20 mg per cent (or about 10 per cent of the total) in nearly all instances.

*Group B*—This group consisted of thirty-one patients with various diseases as noted, excluding known illness due to coronary artery disease. The ages ranged from 24 years to 62 years, the average being 46 years. Twenty patients were men, eleven were women. As shown in Fig 2, miscellaneous patient controls, the average initial cholesterol was 279 mg and the average final cholesterol was 280 milligrams. The intervals of observation ranged from one week to twenty-six weeks, the average being nine weeks. In this group were twelve patients whose serum cholesterol varied less than 10 per cent between the first and last determinations, the average amount of cholesterol variation being 23 milligrams. There also were ten patients whose serum cholesterol values showed substantial decreases, on an average of 83 mg between the first and last determinations. There were an additional nine patients whose serum cholesterol increased on an average of 73 mg between the first and last determinations. In Fig 2, describing this miscellaneous series of conditions, some involving elevated cholesterol values, but in which coronary artery disease was absent, the tendency for greater fluctuations to occur is evident from the graph, and increases or decreases up to 100 mg or more occur as frequently as lesser deviations.

*Group C*—Group C consisted of fifty patients who had had recent coronary artery occlusions (coronary occlusion controls), Fig 3, within six months prior to the study. Their ages ranged from 38 years to 80 years, the average being 62 years. Forty patients were men, ten patients were women. The interval

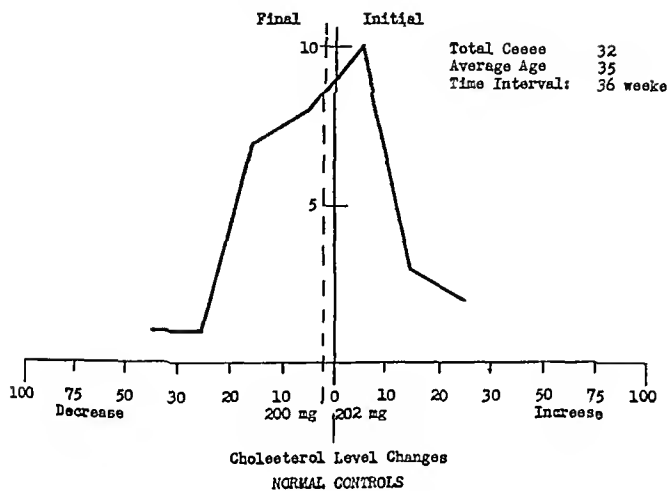
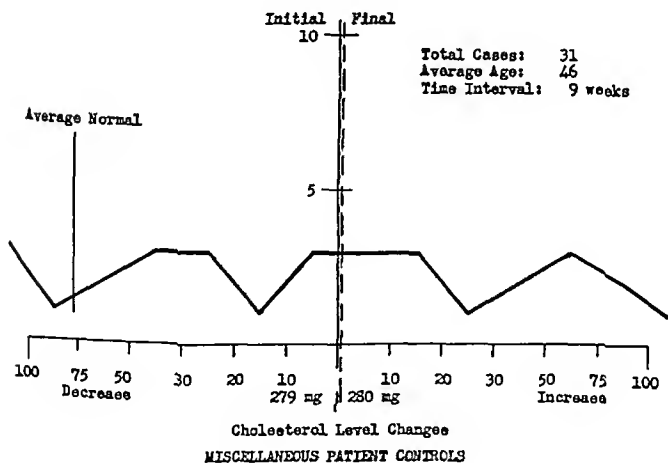
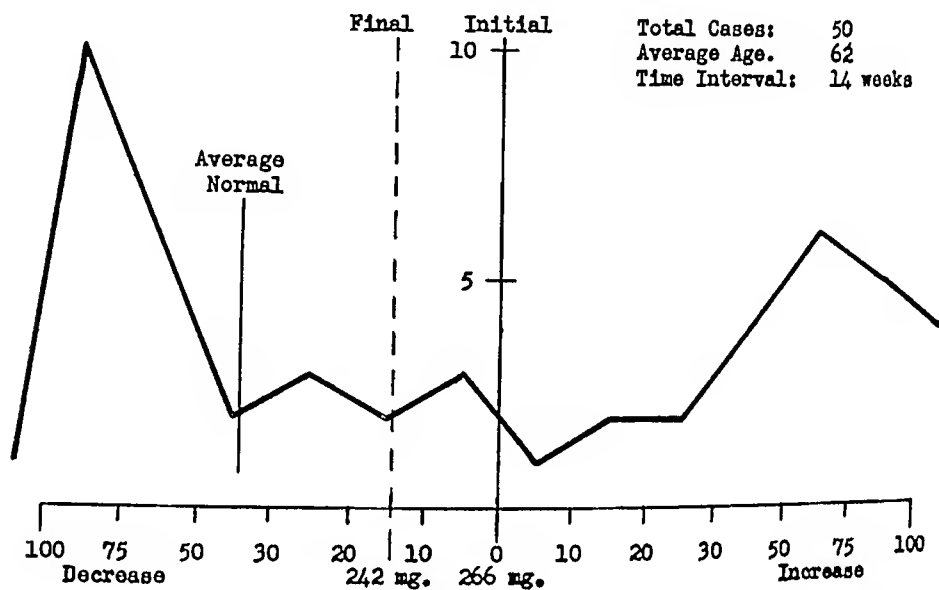


Fig 1



Fig

range between initial and final cholesterol determinations was from one week to sixty weeks, the average being fourteen weeks. In this group there were three further subgroups. Ten patients had less than 10 per cent variations in serum cholesterol from first to last determinations, the average variation being 26 milligrams. Nineteen patients showed substantial interval cholesterol decreases with an average decrease of 69 milligrams. Twenty one revealed interval



Cholesterol Level Changes  
CORONARY OCCLUSION CONTROLS

Fig 3

cholesterol increases with an average of 83 mg increase. Fig 3 (coronary occlusion controls) reveals that the average initial serum cholesterol was 266 mg and the average final serum cholesterol was 242 milligrams. In acute coronary occlusion, which condition is frequently associated with elevated blood cholesterol, as has been shown previously,<sup>2</sup> there is a more marked tendency to fluctuate. Here it is noted that the majority of cases show a marked difference in level from an initial determination to a succeeding one and that this difference may as frequently be an increase as a decrease.

*Group D*—This group consisted of forty-eight patients who had had recent coronary artery occlusion within six months prior to testing and treating, most of whom had received 6 Gm of choline bicarbonate daily. This was given in three divided daily doses, each containing 2 Gm (coronary occlusion—choline therapy, Fig 4). The ages ranged from 29 years to 80 years, with an average age of 59 years. Forty-two patients were men and six patients were women. The interval range between the first and last cholesterol while on choline therapy was from one week to sixty-seven weeks, the average being twelve weeks. This

group had three further subgroups. One consisted of thirteen patients who showed less than 10 per cent interval variation in serum cholesterol values. The average variation was 5 milligrams. Eighteen patients revealed an average interval decrease of 72 mg while on choline therapy. Seventeen patients showed an average interval increase of 98 mg on choline therapy. Fig 4 further shows how these fluctuations render more difficult the interpretation of the effect of

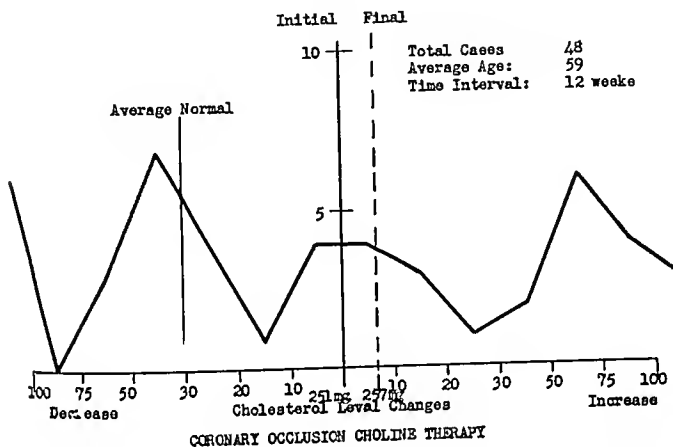


Fig 4

choline feeding on cholesterol levels. However, by graphing the results on this series (Fig 4) in the same manner, it is apparent that no significant change can be attributed to the choline which is not already accounted for by the fluctuations inherent in the disease.

#### DISCUSSION

Confirmation is again made of Sperry's observation that in normal health the blood serum cholesterol is maintained at a constitutional level characteristic for the individual and from which significant alterations are not prone to take place. It is of interest to note here that in two of the normal control subjects wider variations in serum cholesterol were found than in the other subjects. Upon careful interrogation, it was elicited that one subject aged 41 had had a severe attack of infectious hepatitis eighteen years previously. Liver function tests in this individual had shown moderate impairment up to five years following recovery but had not been repeated since the subject felt himself to be in good health. After the serum cholesterol determinations had been made and were found to be more variable than in the other normal subjects (a variation of 29 mg), liver function tests were repeated and showed a somewhat low grade impairment of liver function tests.

The other normal control subject, age 42, who revealed a 38 mg variation, was closely questioned and was found to have a brother who had died recently of an acute coronary occlusion with hypercholesterolemia, one sister who had had a coronary artery occlusion, and parents who had died of coronary occlusions. A familial susceptibility or hereditary cholesterol metabolism disorder associated with coronary artery disease may be a possibility in this case.<sup>13</sup> Our control series of fifty cases of coronary thrombosis (Group C) confirms the findings of Steiner and Domanski<sup>11</sup> who also found that the serum cholesterol level in patients with coronary arteriosclerosis is variable and subject to wide fluctuations. Our finding of wide serum cholesterol fluctuations in miscellaneous diseases is in agreement with the report just published by Astup.<sup>12</sup> The Group D patients with coronary artery occlusions who were under choline therapy revealed the same type of fluctuations of serum cholesterol observed in the control group of noncholine-treated coronary artery occlusion cases. That is to say, there were three similar subgroups, one showing 10 per cent fluctuation in serum cholesterol values on choline treatment, another showing significant increases in serum cholesterol values on choline treatment, and the other showing significant decrease under therapy.

It has been demonstrated that the liver exerts the chief control over cholesterol metabolism, as well as fat metabolism.<sup>1</sup> It also has been established that the liver plays the main role in the synthesis, distribution, and regulation of cholesterol in the blood stream. It is therefore possible that abnormal variations or instability in the serum cholesterol levels encountered in Groups B and C, comprising coronary thrombosis and certain other diseases, indicate an impairment in the function of the liver regulating cholesterol metabolism in these diseases.

#### SUMMARY AND CONCLUSIONS

- 1 In a series of thirty-two normal subjects, the constancy of blood serum cholesterol levels was reaffirmed over prolonged test periods using a modification of the Speery-Schoenheimer serum cholesterol procedure.

- 2 Wide variations in serum cholesterol values were found in a group of thirty-one patients with miscellaneous diseases.

- 3 Marked fluctuations in serum cholesterol values were observed in a series of fifty patients who had recently experienced a coronary artery thrombosis.

- 4 Analogous wide fluctuations in serum cholesterol values were found in a series of forty-eight patients who had recently experienced a coronary artery thrombosis and ingested 6 Gm of choline daily. These fluctuations rendered it impracticable to determine whether choline effected a reduction or increase in serum cholesterol levels.

- 5 It is suggested that variations or instability in serum cholesterol exceeding 15 per cent when determined by the Speery-Schoenheimer procedure or a proved modification thereof in an individual presumed to be normal may possibly indicate a systemic disorder or latent illness.



6 It is suggested that if the Sperry Schoenheimer procedure or a proved modification thereof shows abnormal variations in serum cholesterol, these may be due to a disturbance in the function of the liver regulating cholesterol metabolism

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# IRON METABOLISM

## ERYTHROCYTE IRON TURNOVER

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THE hemoglobin cycle in man is particularly accessible to studies employing radioactive isotopes of iron. The incorporation of radioiron into circulating hemoglobin or the disappearance of tagged cells from circulation may be measured. Such studies have necessarily been of short duration. In the turnover of the red cell mass, iron liberated from hemoglobin is passed on to other tissue and serum proteins (Fig 1) and rapidly rerouted to the bone marrow. Once the radioiron reaches a state of equilibrium in the circulating red cell mass, the level of radioactivity may remain relatively constant for months or years. In the present study it has been possible to isolate the erythrocyte portion of the hemoglobin cycle, so that the survival of a population of red cells of approximately the same age might be determined.

The assumption has been made on the basis of *in vitro* studies<sup>1, 2</sup> that circulating red cells neither take up nor give off iron. The reticulocyte, however, has recently been shown to take up iron and synthesize heme.<sup>3</sup> These studies have allowed further observations *in vivo* and over a long period of time regarding the question of iron exchange between red cell and plasma.

Certain observations have previously been made regarding the breakdown and reutilization of hemoglobin iron. In studies on blood preservation, donors were prepared by injecting radioiron and obtaining blood after the iron had been incorporated in the red cell mass. The disappearance rate of these tagged erythrocytes from the blood stream of the recipient was measured over a period of twenty-four to forty-eight hours.<sup>4, 7</sup> Thereafter, the radioiron was rapidly incorporated into new red cells so that in the ensuing days the level of radioactivity approximated that originally present. If red cell destruction occurred more gradually, no drop in the circulating radioactivity level was observed. This efficient reutilization of broken down hemoglobin iron has been commented on by several investigators as indicating that iron so liberated from hemoglobin falls into a small labile reserve which is rapidly recruited through the hemoglobin cycle.<sup>8, 9, 10</sup> It has been shown that this utilization of radioiron for hemoglobin production may be largely prevented under two circumstances.<sup>11</sup>

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first, when the bone marrow is producing little or no blood and second when the iron stores are greatly enlarged. Advantage was taken of this in the choice of recipients for our studies. Subjects were selected who for one of these reasons should be unable to utilize the radioiron liberated from tagged erythrocytes. It would then be possible to follow the intracellular radioiron through the period of time that the red cell remained in the circulation provided no free exchange of iron occurred.

## PROTEIN-IRON COMPLEXES OF THE HEMOGLOBIN CYCLE

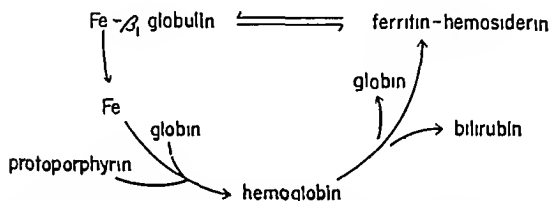


Fig 1—In the serum iron is bound to a beta globulin which transports iron from one tissue to another within the body. In the marrow the iron is stripped from the beta globulin by the developing red cell and synthesized along with pyrrole pigment and globin into hemoglobin. As hemoglobin the red cell iron would appear to remain within the erythrocytes until the stroma is broken down. At this time the pigment is degraded into its component parts and the iron may reappear in the serum bound to the transport globulin or may remain in the tissues incorporated in one of the iron storage proteins ferritin or hemosiderin.

### MATERIALS AND METHODS

Single isotopes of  $\text{Fe}^{55}$  and  $\text{Fe}^{59}$  obtained by cyclotron bombardment at the Massachusetts Institute of Technology were employed in both human and animal studies. Radioactive iron containing both isotopes  $\text{Fe}^{55}$  and  $\text{Fe}^{59}$  with a specific activity of 20 and 80 microcuries per milligram of iron obtained from Oak Ridge was used in some animal experiments. Radioactive donors were prepared as previously described.<sup>2</sup> Tagged blood for transfusion was drawn into acid citrate dextrose preservative and given immediately to the recipient except in one instance in which the effect of storage was under study. The circulating level of radioactivity approximately twenty minutes after blood was given to the recipient was determined and taken to represent 100 per cent of the transfused cells. In two instances, however, where subsequent levels were significantly higher, the latter values were assumed to represent 100 per cent. The blood volume as determined by T1824 dye was compared with the cell volume as determined by dilution of the transfused tagged cells. Subsequent blood samples were taken into calibrated centrifuge tubes over a period of 150 days. Hematocrits were determined on an aliquot by the Wintrobe method. The subsequent wet ashing of samples, addition of carrier iron, precipitation, electroplating and coating have been described previously.<sup>12</sup>

### EXPERIMENTAL DATA

*I Animals*—Dog SFD90 was given 0.2 m.  $\text{Fe}^{55}$  ( $1 \times 10^6$  counts per minute) intravenously and the subsequent incorporation of the iron into the circulating red cell mass was determined (Fig 2). At fifteen days 75 per cent

of the injected iron was found in the peripheral blood\*. On the twenty seventh day and until the 108th day injections of nonradioactive iron in the form of ferrous-ascorbate gelatin† totalling 4,170 mg were given intravenously. Throughout the period of observation the dog remained in good condition.

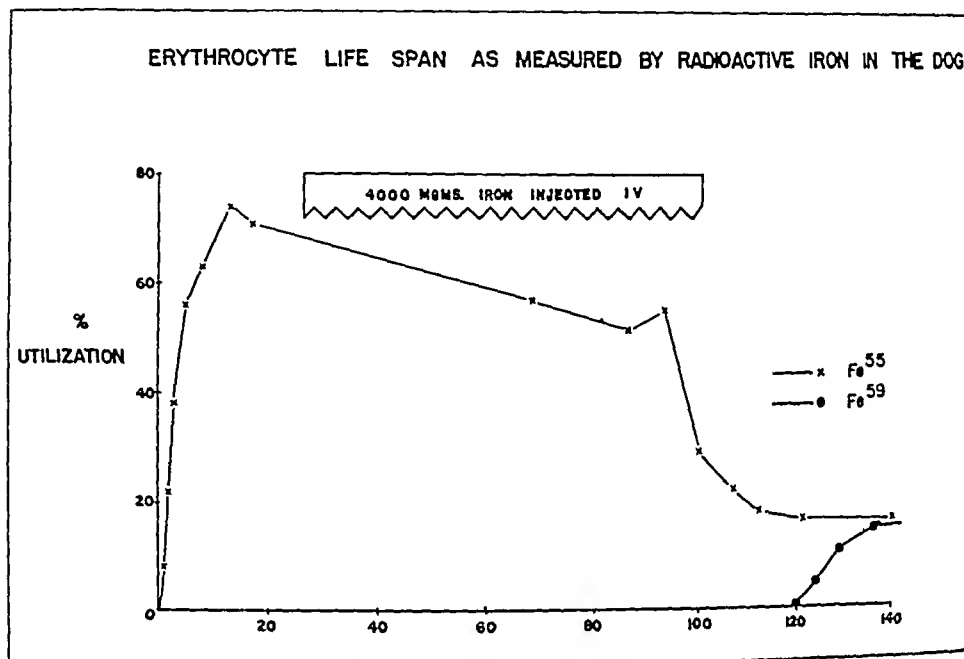


Fig 2—The initial utilization of  $\text{Fe}^{55}$  in a normal dog is shown. Circulating radio levels were followed during a period of 100 days while nonradioactive iron was injected intravenously. A subsequent utilization curve with  $\text{Fe}^{59}$  is shown. The ordinate is expressed in days.

There was a gradual drop in hemoglobin from 18 to 15 grams but no change in cell indices, reticulocytes, or in the leucocyte picture. In Fig 2 is shown the level of circulating radioactivity. At 120 days 0.5 mg of  $\text{Fe}^{59}$  ( $1 \times 10^6$  counts per minute) was injected intravenously. Sixteen per cent of this iron appeared in the red blood cell mass at the end of two and a half weeks.

In two further experiments dogs were placed on corn grit and iron diets which had previously been shown to produce large iron stores in animals<sup>13</sup>. These dogs received red cells prepared by injecting radionon in a single donor dog one week previously. A similar rapid decline in circulating radioactive levels was observed in these dogs, in the neighborhood of 100 days. The red cell life was calculated from the time 50 per cent of the tagged cells had appeared in the blood of the donor dog to the time 50 per cent of the circulating radioactivity had been lost from the blood of the recipient dogs. In Dog J-FD219 the erythrocyte life span was 106 days and the average survival in Dog C-FD218 was 109 days. These dogs showed no significant hematologic

\*The dog's blood volume was determined by T1824 dye and the per cent circulating radioactivity calculated as previously described<sup>11</sup> according to the formula:  $\frac{\text{per cent activity} \times \text{counts per cubic centimeter red cells} \times \text{cubic centimeter circulating red cells}}{\text{counts injected}}$

†This iron preparation was obtained through the courtesy of the Knox Gelatin Products, Inc., Camden, N. J.

change through the period of experimentation. The pathologic examination of both dogs revealed extensive deposits of hemosiderin and greatly increased tissue iron by analysis.

### LIFE SPAN OF ERYTHROCYTES TAGGED BY RADIOIRON

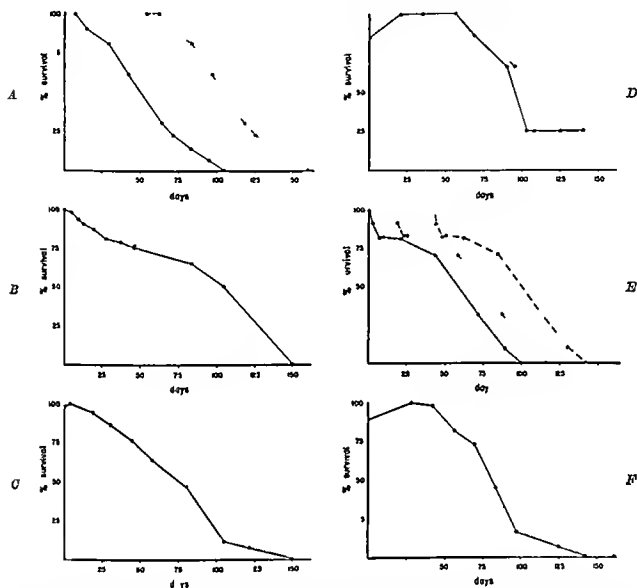


Fig 3—The solid line represents the actual radioactivity levels in the circulating blood of the recipients. The dotted line in *A*, *D*, and *E* allows for the added period of red cell life in the donor. In *E* the third line represents the additional period of blood storage.

*A* Sixty day old normal cells to patient with aplastic anemia.

*B* Pernicious anemia reticulocytes to patient with aplastic anemia.

*C* Iron deficient reticulocytes to patient with aplastic anemia.

*D* Iron deficient erythrocytes to a patient with hemochromatosis.

*E* Twenty-one-day old blood stored for twenty four days transfused to a patient with Cooley's anemia.

*F* Iron deficient reticulocytes transfused to a patient with Cooley's anemia.

**II Human Subjects**—Six populations of radioactively tagged erythrocytes were followed in recipients chosen because of their large iron stores and/or impaired bone marrow function. There follows in each instance a characterization of the blood given, the age of the red cells given, the hematologic status of the recipient, and the radioactive survival curves as determined by measuring levels of radioactivity in the recipient blood. The data are plotted in Fig 3.

**1** Five hundred cubic centimeters of blood were drawn from a young man who had been given radionon ( $\text{Fe}^{59}$ ) sixty five days previously. The donor's red cells were normal in appearance and measurements showed a mean

corpuscular volume (MCV) of 85, a mean corpuscular hemoglobin (MCH) of 32, and a mean corpuscular hemoglobin concentration (MCHC) of 35. The recipient W-FD129 was a girl of 6 years with aplastic anemia who had been transfused every six to eight weeks since the age of 2 months. Preceding the transfusion her blood showed a hemoglobin of 5 grams, red count 1.8 million, and hematocrit 16 (MCV 108, MCHC 28, MCH 31) and no reticulocytes in 1,000 red blood cells. The subsequent decline in circulating radioactivity levels is shown in Fig 3, *A*. The projected life span of the red cells, allowing for the time they had circulated in the donor, is shown by the dotted line.

*B* The same recipient, W-FD129, was transfused one month later with 250 cc of blood tagged with  $\text{Fe}^{59}$ . The donor in this case, G-FD131, had classical pernicious anemia and had received liver six days previously and radionon four days before the blood was drawn. The transfused blood had a MCV of 130, MCH 37, and MCHC 35. There were 31.9 reticulocytes. In the blood fractionated by albumin flotation<sup>14</sup> the distribution of radioactivity corresponds to the distribution of reticulocytes (Table I). These data would indicate that the isotope is found almost exclusively in the reticulocyte fraction and that, in effect, tagged reticulocytes are being transfused. Subsequent survival of this blood is shown in Fig 3, *B*.

*C* Recipient W-FD129 was used for a third study after all previously administered radioactivity had disappeared from her circulating blood. Donor SL-FD262 had typical untreated polycythemia vera. The donor's blood showed a hemoglobin of 20.1 grams with slight microcytosis and hypochromia (MCV 75, MCH 23, MCHC 31). Five hundred cubic centimeters of blood were drawn on the third day after injection of radionon. Evidence that these tagged cells were comprised chiefly of reticulocytes is shown in Table I. The determinations of circulating radioiron levels in the recipient are shown in Fig 3, *C*.

*D* Five hundred cubic centimeters of blood were drawn from the same donor, SL-FD262, on the tenth day following injection of radionon. This blood was transfused to recipient R-FD108 who had hemochromatosis confirmed by

TABLE I

IRON DEFICIENT BLOOD	COUNTS/UNIT CELLS	RETICS (%)
Top fraction	2,900	10.8
Middle fraction	920	2.9
Bottom fraction	500	1
PERNICIOUS ANEMIA BLOOD		
Top fraction	5,660	39.1
Bottom fraction	187	0.1

liver biopsy. The recipient had a saturated non binding protein, a serum iron of 250  $\mu\text{g}$  per 100 cc, and a slightly macrocytic anemia (hematocrit 40, MCV 102). Radioactivity was expressed as counts per cubic centimeter of red cells since it was felt that the red cell mass should be relatively constant through the period of observation. This is in contrast to the other cell viability experiments in which activity is related to cubic centimeters of whole blood. There is no evident explanation for the initial lower level of radioactivity unless it is

related to the temporary red cell mass increase due to the transfusion. It is to be noticed (Fig 3, *D*) that there is a reutilization in this patient of 25 per cent of the erythrocyte non similar to that seen in previous animal experiments.

*E* A third phlebotomy was performed on the donor on the twenty first day after iron injection. This blood was preserved in acid citrate dextrose for twenty five days at 4° to 6° C and then transfused. The recipient JS FD265, was a 4 year-old child who had Cooley's anemia and had received approximately twenty transfusions before the present study. On the day of transfusion his hemoglobin was 10 grams. The survival time of these red cells and the projected survival allowing for their time in the donor and preservative are shown in Fig 3, *E*.

*F* Donor LK FD244 was a patient with polycythemia vera who had been controlled by phlebotomy for two years. He showed a high grade iron deficiency at the time of phlebotomy (MCV 70 MCH 22 MCHC 30). Six days after injection of  $\text{Fe}^{59}$  500 cc of blood were removed and transfused immediately to recipient L FD263. The subsequent levels of circulating radio iron per cubic centimeter of whole blood are shown in Fig 3 *F*. The recipient in this transfusion was a 4 year old girl who had been anemic since birth with typical findings of Cooley's anemia. She had had more than eight transfusions before the present study and a hematocrit of 18.2 prior to transfusion.

In another series of observations, normal male subjects were given  $\text{Fe}^{59}$  intravenously and beginning twenty one days later iron was taken for four to six months by mouth immediately after meals. Subjects T and R consumed 200 Gm of ferrous sulfate and Subject M 400 grams. It would appear that enough iron was absorbed in this fashion to interfere somewhat with reutilization and to allow one to approximate the life span of the erythrocyte in each case (Fig 4). Mean cell age was calculated as 125 days (Subject T) 125 days (Subject R) and 114 days (Subject M).

#### DISCUSSION

The sensitivity of the counters used reach 3 per cent efficiency in the case of  $\text{Fe}^{59}$  and 20 per cent in the case of  $\text{Fe}^{50}$ . The counting levels obtained from blood samples were sufficient to allow accurate measurement of samples containing less than 5 per cent of the initial circulating radioactivity except for experiment B (Fig 3 *B*). Here accurate counting that is counts more than twice background, was attained only in values showing over 30 per cent erythrocyte survival.

In the dogs and in the patient with hemochromatosis (Figs 2 and 3 *D*) there was appreciable reutilization of the erythrocyte non in the neighborhood of 25 per cent. Since these new base lines are stable over a period of several weeks it seems justifiable to assume 100 per cent destruction of transfused erythrocytes when this level has been attained. In the remaining transfusions there was no significant reutilization. Normal subjects fed iron however showed so great a reutilization (approximately 75 per cent) that interpretation of the data is difficult.

In these studies a population of red cells of approximately one age has been followed throughout their survival period in the circulating blood. The entrance of these cells into the circulation in the donor is shown by the utilization of the injected non for hemoglobin production. In Fig 5 is a composite curve of such utilization of radioiron obtained previously in normal subjects. This

#### INFLUENCE OF ORAL IRON ADMINISTRATION ON RADIOIRON LEVEL OF THE BLOOD

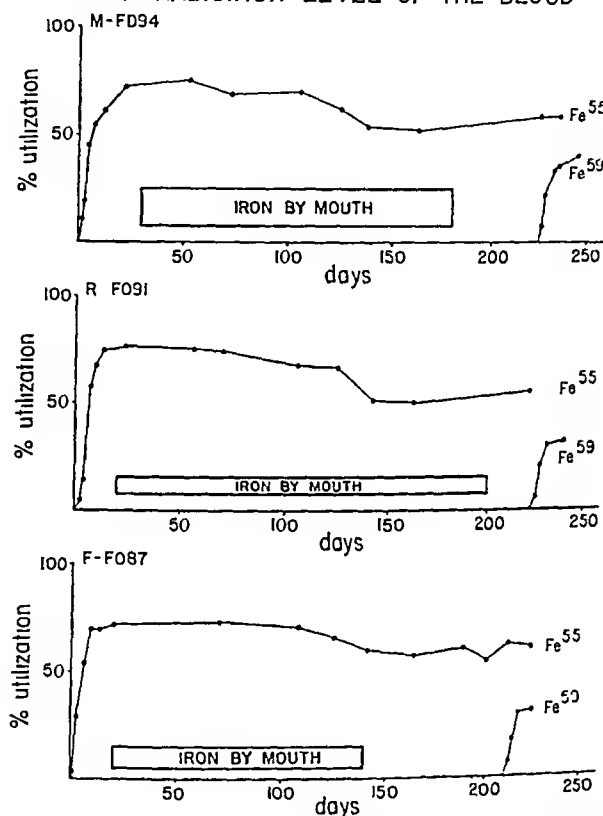


Fig 4—The initial utilization of radioiron in three normal subjects is shown and the subsequent circulating radioactive levels during the period of iron intake by mouth. The subsequent utilization curve with Fe<sup>59</sup> is also shown. It will be observed that between 100 and 150 days there is a significant decline in circulating radioactivity levels in each subject.

indicates that normally the fourth day will represent the mean age of tagged cells and that some 75 per cent of the total number of tagged cells appear in circulation between the second and seventh days.<sup>11</sup> In non deficient and pernicious anemia patients responding to treatment, the utilization may be even more rapid. For purposes of calculation in Fig 3, corrections are made by a dotted line for the mean age of the cells, according to when they would be expected to appear in the donor's circulation.

It might be questioned whether the sample taken immediately after transfusion is representative of the initial red cell level or whether during the process of transfusion which took between one-half and two hours some red cell-



might already be lost from the blood stream. It has been previously demonstrated that nonviable erythrocytes may disappear very rapidly from circulation, that is, within minutes of transfusion.<sup>4, 15</sup> For this reason it appeared necessary to have some check on the initial level. Highly quantitative measurements were not possible since the blood given was only roughly measured and

### UTILIZATION OF RADIOIRON FOR HEMOGLOBIN PRODUCTION

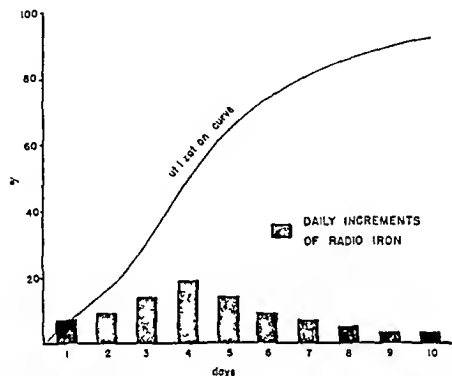


Fig. 5—On the basis of data previously reported a composite utilization curve is shown. This has been normalized from the 4 per cent which appears in the blood to 100 per cent so that the daily increment of radioiron incorporated in erythrocytes may be shown.

since the transfusion set was not always completely emptied of blood. In patient FW FD129 three transfusions were given (I, 3, 1 B and C). The calculated blood volume on the basis of the radioactivity administered and the initial post transfusion level were respectively in observations A, B and C 1340 cc, 1400 cc and 1280 cubic centimeters. Since A represents fresh normal cells which have been shown previously to be a reliable measure of the circulating mass,<sup>10</sup> the immediate survival of the other two populations of red cells may be assumed to be reasonably good. In D the calculated radioactive cell volume was 2160 cc while the Evans blue dye determination gave a cell mass of 1975 cubic centimeters. In E a blood volume employing T1824 immediately after transfusion gave a determination of 1030 cc whereas the radioisotope blood volume was 950 cubic centimeters. In patient L FD263 (F) an Evans blue blood volume performed six months after the initial study was 1500 cc as compared with the post transfusion radioactive measurement of 1200 cc in the same patient. In no instance therefore was there an indication of an abnormally large blood volume by radioactive measurement using the twenty minute samples. This would appear to exclude any appreciable cell destruction during the time of transfusion since such an event would result in a greatly enlarged cell volume determination by the radioisotope method.<sup>15</sup>

Previous work has shown that very little radionon injected intravenously appears in the circulating red cell mass when bone marrow synthesis is severely impaired, suggesting that only as a result of hemoglobin synthesis by the developing red cell can radionon gain access to the erythrocyte<sup>10, 11</sup>. In the present studies, reutilization of iron is largely or completely blocked. Should there be an exchange of hemoglobin iron with plasma iron, the level of circulating activity would rapidly fall. The radioactivity in the recipients' circulation, however, followed a survival curve similar to that obtained when the pyridol pigment<sup>17</sup> or globin of the hemoglobin molecule is isotopically tagged. It would then appear to be a safe assumption that the mature red cell throughout its life span has no appreciable exchange of iron with its surrounding environment.

In three dogs the average turnover of iron from the time of appearance in circulation to disappearance is from 95 to 109 days. In patients transfused with various populations of erythrocytes, 50 per cent of the erythrocytes had disappeared from circulation between 77 and 110 days after their appearance in circulation. The one normal cell population (Fig 3, A) showed a mean cell survival of 110 days. This is consistent with other data of the life of the human red cell<sup>18</sup> and would indicate that normally there is approximately a 1 per cent daily turnover of iron through the red cell mass as a result of erythropoiesis and erythrocyte breakdown.

In contrast to differential agglutination studies in which the red cell population measured is a mixed one, in these studies red cells of a single age could be followed throughout their life period. By drawing blood shortly after injection of radionon it was possible to obtain young cells as shown in Fig 3, B and C. That these young cells represent reticulocytes is indicated by Table I and by other studies,<sup>19</sup> demonstrating that in fractionated blood the reticulocyte level and radioactive level show a high degree of correlation. Other studies<sup>3, 20</sup> have demonstrated that reticulocytes are capable of synthesizing heme and maturing, and that their disappearance from circulation is associated with an increase in mature red cells. The present observations would indicate that they not only mature but have a reasonably normal life span.

It is of interest that in pernicious anemia the reticulocytes after liver therapy have a normal life expectancy, since other observations on mixed populations of untreated pernicious anemia macrocytes have indicated a considerably shorter life span. Iron deficiency reticulocytes as shown in Fig 3, C and F survive a somewhat shorter than normal period, whereas the same cells in a subject with a relatively normal blood picture show a relatively normal survival. This suggests an influence of anemia per se on the life span although the varied nature of the recipients is such that no conclusions can be drawn. The different survival curves obtained with red cells from the same donor in different recipients (Fig 3, C and D) emphasize the importance of such factors. It is of interest that storage of blood before transfusion seemed to result in an immediate loss of approximately 20 per cent of the transfused red cells while the remaining erythrocyte population survived what would appear to be a relatively normal life span.

In subjects fed non the erythrocytes remained in their normal environment and were subjected to no manipulation. Although the great reutilization obscures the erythrocyte breakdown the mean cell age estimated at 122 days is quite similar to the figure of 127 days obtained by Shemin and Rittenberg<sup>1</sup> using  $N^{15}$  in the same type of study.

#### CONCLUSIONS

The reutilization of radioactive iron from broken down red cells is effectively blocked by the presence of enlarged iron stores and/or bone marrow dysfunction in animals and in human subjects. By using recipients with these characteristics, the erythrocyte unit of iron metabolism may be isolated.

Under these experimental conditions it was possible to measure the life span of a red cell population of a single age. Observations were made of tagged macrocytes, microcytes and normal red cells in normal and anemic recipients. The reticulocytes from a patient with pernicious anemia responding to therapy and from a patient with iron deficiency have been shown to have a relatively normal life span.

The erythrocyte iron turnover measured directly is approximately 1 per cent a day in dog and in man.

There is no discernible exchange of iron between the red cell and its surroundings throughout the life span of the erythrocyte.

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## TYROSINE METABOLISM IN HUMAN SCURVY

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### INTRODUCTION

IT HAS been shown that vitamin C is intimately connected with the metabolism of tyrosine and phenylalanine. Sealock and Silberstein<sup>1</sup> reported that when tyrosine was fed to scorbutic guinea pigs homogentisic acid was excreted, a phenomenon that could be prevented by the administration of l ascorbic acid. They also demonstrated<sup>2</sup> that tyrosine, fed to guinea pigs on a diet deficient in vitamin C, resulted in the excretion of homogentisic, *p* hydroxyphenylpyruvic, and *p* hydroxyphenyl lactic acids, and that the administration of l ascorbic acid prevented the abnormal excretion of these metabolites. Painter and Silva<sup>3</sup> fed large doses of tyrosine to scorbutic guinea pigs but found no excretion of homogentisic acid in the urine but did observe excessive excretion of the intermediary metabolites of tyrosine. In addition, Levine and co-workers<sup>4</sup> noted that premature infants fed diets of vitamin C free cows milk containing 5 Gm. or more of protein per kilogram of body weight excreted *p* hydroxyphenyl lactic and *p* hydroxyphenylpyruvic acids the excretion of which was eradicated by the administration of ascorbic acid.

The purpose of this communication is to report the effects in normal and scorbutic human beings of feeding large amounts of tyrosine and the rapid effect of l ascorbic acid in correcting the abnormal tyrosine metabolism found in the individuals with scurvy.

### MATERIALS AND METHODS

The patients observed in this study were four scorbutic men aged 62, 64, 66, and 71 years, two normal men aged 22 and 29, and one normal woman aged 75.

During the course of the studies the scorbutic patients were maintained on a vitamin C free diet consisting of two quarts of boiled skimmed milk, 100 grams of boiled rice and thirty six soda crackers daily. Sugar coffee and vitamin C free purified jellies were allowed ad libitum. The two normal male subjects were allowed their usual diets. The normal female subject was maintained on the same vitamin C free diet as the scorbutic patients but received large supplements of crystalline ascorbic acid. Twenty four hour samples of urine were collected daily from the patients and after suitable control periods of at least six days, all except one scorbutic patient were given 20 Gm. of powdered L tyrosine per day orally divided into four doses of 5 Gm. each.

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‡This 5 year-old patient was thought to have scurvy because of purpura. The purpura was of senile type only and he was then studied as a normal patient after receiving large supplements of ascorbic acid.

Kindly supplied by Merck & Co., Inc., Rahway, N. J.

After a period of tyrosine administration, four of the scorbutic patients received large doses of ascorbic acid in addition to the tyrosine. When possible, a control period was included after the discontinuance of tyrosine.

The ascorbic acid content of the white blood cell platelet layer was determined by the method of Butler and Cushman.<sup>6</sup> The addition of 100 mg of human fibrinogen (Fraction I of human plasma [Cohn]<sup>7</sup>) to 10 ml of oxalated blood produced better separation of the white cell platelet layer for assay. The whole blood ascorbic acid levels were determined by the method of Keuther.<sup>8</sup>

The total excretion of hydroxyphenyl compounds in the urine, expressed as tyrosine equivalents, was measured by the method of Medes.<sup>9</sup> The compounds measured by this method include tyrosine, *p*-hydroxyphenyl lactic, and *p*-hydroxyphenylpyruvic acids, and are designated "tyrosyl derivatives."

The reduction of phosphomolybdic acid by the urine as outlined by Medes<sup>9</sup> also was determined. This method, for the most part, measures the excretion of *p*-hydroxyphenyl pyruvic acid, but is not specific and will react with homogentisic acid and other reducing substances. Since the reducing substances excreted by the scorbutic patients, while receiving tyrosine, were not chemically identified, their reducing power was calculated as equivalent of a standard solution of hydroquinone. The presence of homogentisic acid was tested for qualitatively by adding ferric chloride to urine that was made alkaline with sodium hydroxide. Calibration curves with hydroquinone standards for the phosphomolybdic reducing power and *l*-tyrosine for the "tyrosyl" derivatives were prepared using the Evelyn photoelectric cell colorimeter. The tyrosyl derivatives were read in the colorimeter exactly one minute after color formation to obtain maximum values. If they were read after allowing the color reaction to proceed for twenty minutes, as outlined by Levine and associates,<sup>4</sup> there was a marked drop in color intensity. The phosphomolybdic reaction was allowed to proceed for three hours before readings were made in the colorimeter.

## RESULTS

**Scorbutic Patients—Case 1** (Fig 1), a 62 year old man, was admitted to the Boston City Hospital because of pain in the right thigh. He had classical signs of scurvy: a positive tourniquet test, purpura on the legs, ecchymoses of the right thigh, and hematomas around earious teeth. There was no detectable ascorbic acid present in the white blood cell platelet layer of the blood. In a control period of six days on a vitamin C free diet, this patient's total "tyrosyl" excretion averaged 195 mg of tyrosine equivalents per day with a range of from 123 mg to 248 mg per day. In this same control period, the excretion of reducing substances as measured by the reduction of phosphomolybdic acid was equivalent to an average of 84 mg of hydroquinone per day and ranged from 37 mg to 146 mg per day.

When the patient was given 20 Gm of tyrosine daily, for six days, the excretion of "tyrosyl" compounds immediately increased, and on the sixth day of administration reached a level of 6,300 milligrams. The reducing substances of the urine remained unchanged from the control values for three days, but then steadily increased until on the sixth day of tyrosine administration the concentration was equivalent to 6,300 mg of hydroquinone per day.

The patient was then given the daily addition of 1 Gm of *l*-ascorbic acid orally as well as 20 Gm of tyrosine per day for five days. The ascorbic acid was then reduced to 0.5 Gm per day for the remainder of the study. With ascorbic acid therapy, the excretion of "tyrosyl" compounds immediately decreased and on the second day of therapy was 53 mg and fluctuated between 453 and 1,073 mg per day for the next seven days. The study ended. The reducing substances of the urine showed no decrease on the first day of ascorbic acid therapy and, in fact, increased to 6,620 mg of hydroquinone equivalent. However, on the second day of ascorbic acid therapy, the concentration of reducing substances dropped to slightly above control levels, averaged 152 mg per day, and remained

it that value for four days. At this time the reducing power increased to a daily average of 252 mg of hydroquinone equivalents and is interpreted as an increased excretion of ascorbic acid since this compound will reduce phosphomolybdic acid under the conditions used.

During the period of tyrosine administration, the capillary fragility increased as measured by the tourniquet test, and there was some bleeding from the gingival hematomas. All signs of capillary fragility disappeared promptly after administration of ascorbic acid.

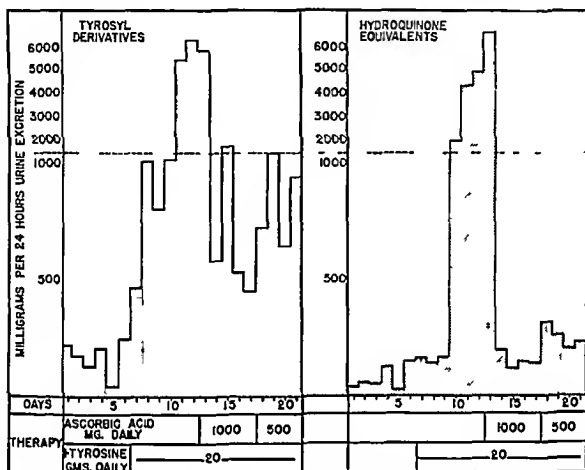


Fig. 1—Case 1 (scurvy). Twenty-four hour excretion levels before and after treatment.

Case 2 (Figs. 2 and 3) was a 64-year-old man with the clinical picture of scurvy and with no detectable ascorbic acid in the white cell platelet layer. He was maintained on the same vitamin C-free diet as provided for the first patient. In a nine-day control period he excreted a daily average of 109 mg of tyrosyl compounds. The reducing substance of the urine in this same control period was equivalent to a daily average of 74 mg of hydroquinone.

The patient was then given 20 Gm of tyrosine daily orally with a large immediate rise in excretion of "tyrosyl" derivatives. On the second day he excreted 7,700 mg and on the fifth day 16,120 milligrams. On the seventh day of tyrosine administration, he was given 2 Gm of ascorbic acid orally per day. This dose was continued for five days and then decreased to 1 Gm. per day for the remainder of the observation period. Following the administration of ascorbic acid the excretion of tyrosyl compounds rapidly fell and, although the patient received tyrosine for twenty-five days after institution of antiscorbutic therapy, his excretion of tyrosyl derivatives after the initial drop became evident averaged only 336 mg per day with a daily range of from 200 to 1,400 milligrams. Tyrosine was stopped for a period of one week during which time the average excretion dropped to 30 mg per day. In order that the patient should act as his own control he was given tyrosine for another eight-day period during which time the average excretion of tyrosyl derivatives was 312 mg per day with the daily maximum being 400 mg on the fourth day. In a final control period of ten days without tyrosine he excreted an average of 110 mg per day.

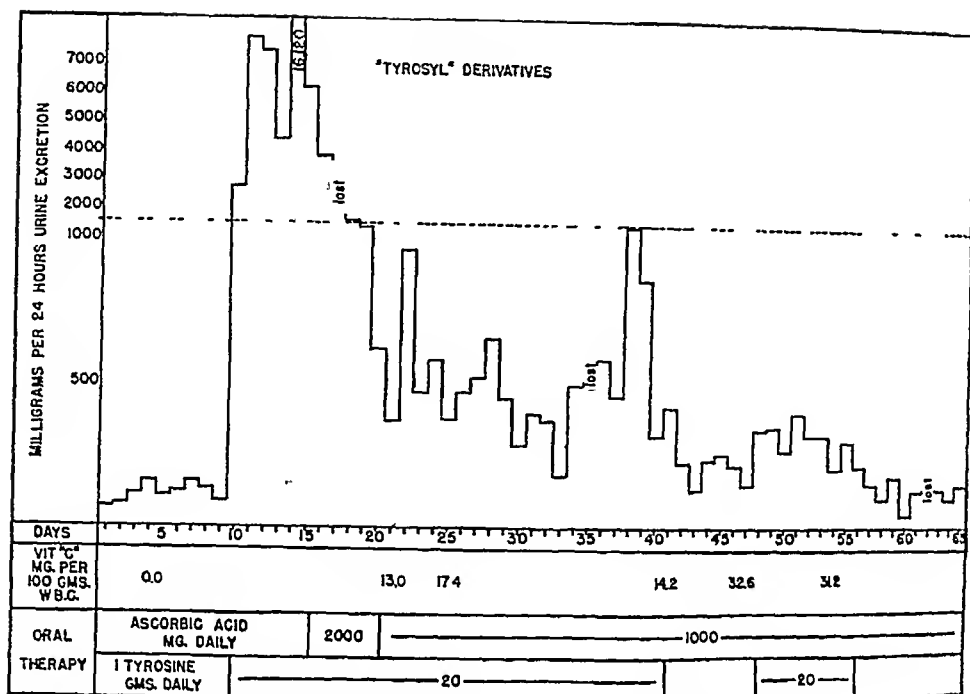


Fig 2—Case 2 (scurvy) Daily 'tyrosyl' excretion

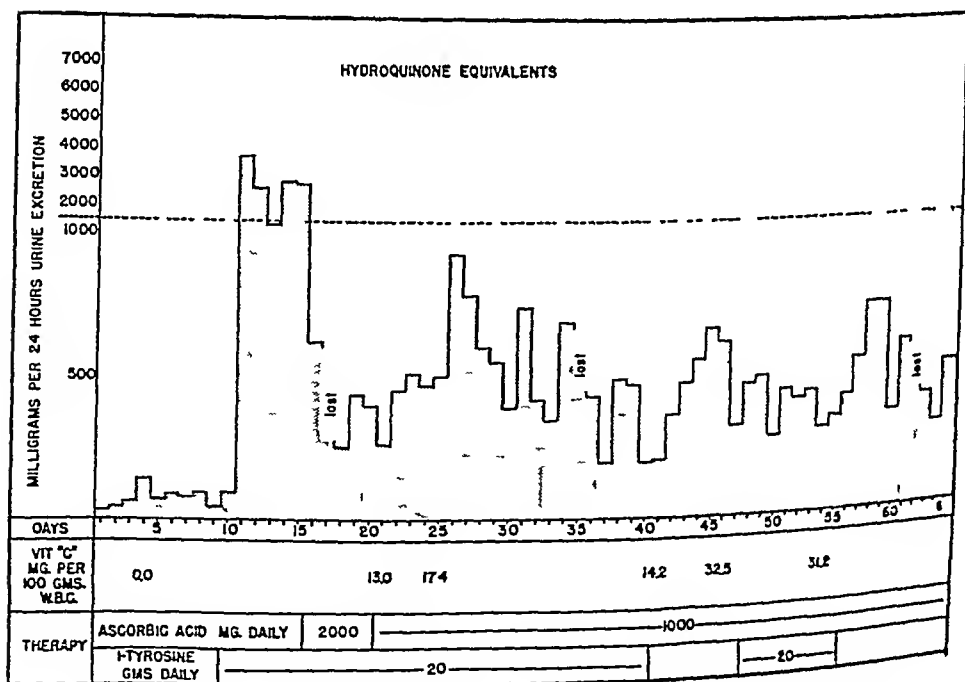


Fig 3—Case 2 (scurvy) Reducing substance in the urine measured as hydroquinone equivalents



The reducing substances in this patient's urine, during the administration of tyrosine increased rapidly to 3,480 mg of hydroquinone equivalents on the second day and ranged above 2500 mg per day throughout this period except for one value of 1,340 mg which may be in part an incomplete sample. There was a marked fall concomitant with the administration of 2 Gm of ascorbic acid per day to an average level of 471 mg of hydroquinone per day. Again it is believed that this higher level of excretion than in the original control period was caused by the excretion of ascorbic acid and was not due to the administration of tyrosine. This contention is borne out by the fact that when tyrosine administration was discontinued for seven days the daily average remained essentially the same (446 mg of hydroquinone equivalents per day) and that on reinstitution of tyrosine administration the level tended to fall slightly averaging 392 mg of hydroquinone per day.

During the period of tyrosine administration, there was a marked increase in capillary fragility. For the first time, the tourniquet test produced numerous small hematomas over the forearm as well as petechial hemorrhages. The patient became weak and developed nausea and diarrhea, all symptoms disappeared when ascorbic acid was given.

*Case 3* (Table I) was a 71 year old man who entered the hospital with hematomas of both calves and marked capillary fragility. There was no detectable ascorbic acid in the white blood cell platelet layer. Throughout the study the patient was on a vitamin C free diet and received no additional tyrosine.

In a six day control period before the administration of ascorbic acid the patient excreted an average of 201 mg of hydroxyphenyl compounds per day and the reducing substance of the urine was equivalent to 64 mg of hydroquinone per twenty four hours. After this control period the patient was given 1 Gm of ascorbic acid a day orally for five days and then 0.5 Gm for the remainder of the study. With ascorbic acid therapy there was no significant change in excretion of tyrosyl derivatives which averaged 196 mg per day. The reducing substances in the urine remained low (averaging 92 mg hydroquinone equivalents per day) for four days and then rose to an average excretion of 192 mg per day which undoubtedly reflected the increased amounts of ascorbic acid being excreted in the urine.

TABLE I CASE 3 (SCURVY) EXCRETION LEVELS BEFORE AND AFTER ADMINISTRATION OF ASCORBIC ACID

TREATMENT	DAYS	AVERAGE TYROSYL DERIVATIVES (MG /24 HR.)	AVERAGE HYDRO QUINONE EQUIVALENTS (MG /24 HR.)
0	6	201	64
Ascorbic acid			
10 Gm. 5 days	10	190	192
0.5 Gm. 5 days			

*Case 4* (Fig 4) The patient entered the hospital complaining of weakness and inability to walk. He had large hematomas in the right popliteal fossa extending to the groin. There was no measurable vitamin C in the white blood cell platelet layer of the blood. Throughout the observations the patient was maintained on the vitamin C free diet. During a five day control period the patient excreted a daily average of 219 mg of 'tyrosyl' derivatives. The average daily excretion of reducing substances in the urine was 20 mg hydroquinone equivalents.

The patient was then given 20 Gm of tyrosine daily. By the third day the 'tyrosyl' excretion had increased to 11300 mg and the reducing substances to 3,075 milligrams.

During the period of tyrosine administration the patient developed nausea, diarrhea, vomiting and marked lassitude. The diet intake was not maintained and urine collections could not be made. Tyrosine was discontinued and the patient received 15 Gm of ascorbic acid parenterally and 25 Gm orally daily. Despite the large doses of ascorbic acid and parenteral dextrose solution the patient did not respond. He died five days after the onset of ascorbic acid therapy in an episode of pulmonary edema and shock. Post mortem examination revealed only pulmonary congestion and no hemorrhagic phenomena.

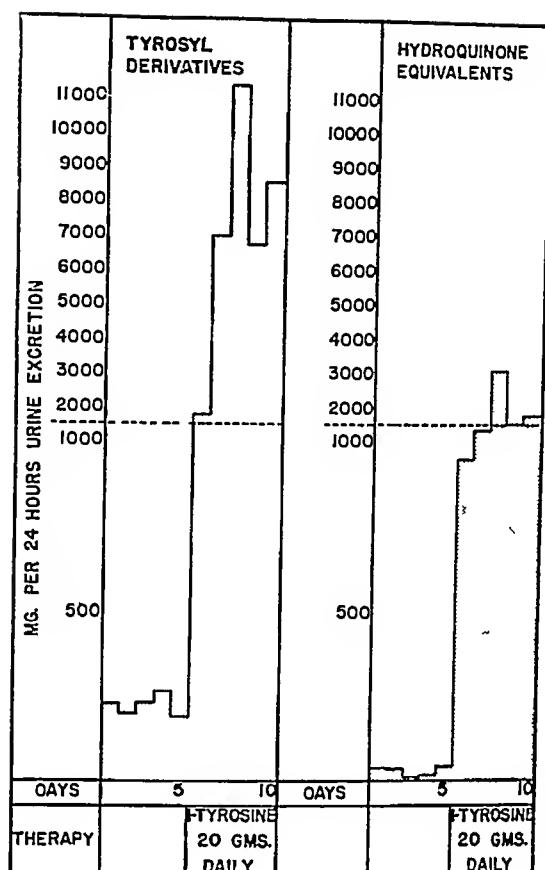


FIG 4—Case 4 (scurvy) Excretion levels during tyrosine administration

TABLE II RESPONSE OF THREE NORMAL SUBJECTS TO 20 GM PER DAY OF L TYROSINE ORALLY

PATIENT	L TYROSINE (GM/DAY ORALLY)	AVERAGE TYROSYL DERIVA TIVE (MG/24 HR)	AVERAGE HYDRO QUINONE EQUIVALENTS (MG/24 HR)	DAYS IN PERIOD
Normal Subject 1	0	242	184	14
Period 1	20	755	167	11
(See Fig 5)	0	287	135	0
Period 2*	0	328	533†	6
	20	734	588†	7
	0	290	171	5
	0	175	99	0
Normal Subject 2	20	614	112	9
	0	134	91	5
	0	218	683†	9
Normal Subject 3	20	582	887†	5
	0	230	636†	5

\*500 mg ascorbic acid given orally during control and administration of tyrosine

†These values are markedly elevated because of ascorbic acid administration throughout this period

*Normal Patients*—*Normal Subject 1* (Fig 5) in a control period of fourteen days excreted an average of 242 mg of tyrosyl compounds per day. The reducing substances in the urine in this same period were 184 mg hydroquinone equivalents per day. He then received 20 Gm of tyrosine daily for eleven days which resulted in a gradually increased excretion of 'tyrosyl' compounds until the fifth day when it was 1,270 milligrams. Thereafter for the next five days a moderate fall in the excretion of 'tyrosyl' derivatives occurred, followed by a second peak reaching 1,018 mg on the last day of tyrosine administration.

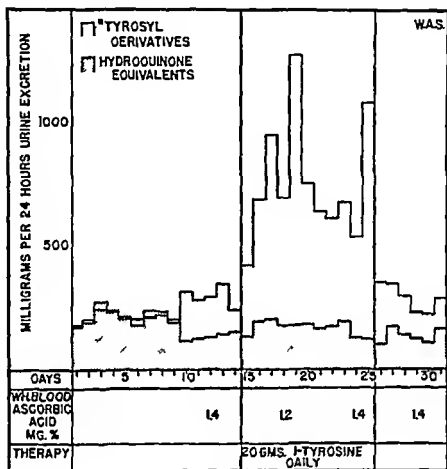


Fig 5—Normal Subject 1 Excretion levels of normal subject during tyrosine administration

tion The average excretion of tyrosyl derivatives during the entire period of tyrosine administration was 755 mg per day in a subsequent control period without tyrosine the excretion dropped to preadministration levels

The reducing substances in this individual's urine did not change appreciably during any portion of the experiment. During the administration of tyrosine they were 167 mg hydroquinone equivalents per day which was slightly lower than in the previous control period. Whole blood ascorbic acid levels were within normal limits and showed no significant change during the control periods or when the patient was receiving tyrosine.

In order to determine if added ascorbic acid would change the response to 20 Gm of tyrosine per day, the same observations were repeated on this patient with the exception that he was given 500 mg of ascorbic acid daily starting three days before the administration of tyrosine and continuing through the entire period of tyrosine administration Table II, Period 2. The addition of ascorbic acid in this instance did not change the response to tyrosine as evidenced by the fact that on one day the patient excreted 1134 mg of 'tyrosyl' derivatives and the average excretion during tyrosine administration was 734 mg per day as compared with the value of 700 mg per day when he was not receiving ascorbic acid. The reducing substances in the urine immediately increased when he took ascorbic acid, but were not augmented further on addition of tyrosine to his diet.

*Normal Subject 2* The response to 20 Gm of tyrosine daily for eight days in another normal male, aged 22, is shown in Table II. It is similar to that of the previously described normal person, although this patient excreted slightly less hydroxyphenyl compounds, averaging 614 mg per day during the period of tyrosine administration. As in the first normal subject, after an initial rise, there was a decreased excretion for three to four days followed by a second rise.

*Normal Subject 3*, a 75 year old woman, was given the same vitamin C free diet as described for the scorbutic patients. In addition, she received 1 Gm of ascorbic acid orally for the first eleven days of control observation and 2 Gm daily throughout the remainder of the study. During a control period of nine days she averaged 218 mg per day of "tyrosyl" derivatives. She was then given 20 Gm of tyrosine daily for a five day period and the "tyrosyl" excretion averaged 582 mg per day. During another five day control period the excretion averaged 230 mg per day.

During a nine day control period while receiving 2 Gm of ascorbic acid daily, the patient's average excretion of reducing substances was 683 mg hydroquinone equivalent. During the period of tyrosine administration it rose to a daily average of 887 milligram. In the second five-day control period the daily average was 636 milligrams. Again it is believed that the high values in the control period are associated with the reducing power of ascorbic acid. This normal subject showed a rise in reducing power of the urine during tyrosine administration. Although no complete explanation is available, the large dosage of this amino acid may have materially aided in the excretion of ascorbic acid.

#### DISCUSSION

From the foregoing results, it would seem safe to assume that the relationship between ascorbic acid and the metabolism of tyrosine in the adult human being is similar to that which had previously been demonstrated in the guinea pig<sup>1,2</sup> and the premature infant.<sup>5</sup> Sealock<sup>1</sup> has reported that normal human beings on a "diet practically free of ascorbic acid" excrete significant amounts of homogentisic acid when ingesting tyrosine, which was prevented by "reasonably large doses of crystalline ascorbic acid." However, no quantitative data are presented as to the amount of tyrosine ingested, the excretion of homogentisic acid, or the amount of ascorbic acid given.

In the present observations, the ingestion of tyrosine orally by scorbutic patients resulted in a markedly increased excretion of "tyrosyl" derivatives. The method used for the detection of the "tyrosyl" compounds theoretically measures tyrosine, *p*-hydroxyphenylpyruvic acid, and *p*-hydroxyphenyl lactic acid. As the urines were not fractionated or the compounds isolated, it is impossible to say which ones were proportionally responsible for the increased excretion of hydroxyphenyl compounds. Evidence that a large proportion was due to *p*-hydroxyphenylpyruvic acid is found in the marked increase of the reducing power of the urine. However, this reaction is not specific since another intermediary of tyrosine metabolism, namely homogentisic acid, will cause the reduction of phosphomolybdic acid. Although qualitative tests for homogentisic acid in these urines were negative, it conceivably could have been present and could have affected the quantitative reduction of phosphomolybdic acid, but could not be detected qualitatively. The absence of homogentisic acid is more in keeping with the animal studies of Panter and Silva.<sup>3</sup>

In the scorbutic patients, the increased power of the urine to reduce phosphomolybdic acid lagged behind the increased excretion of 'tyrosyl' derivatives, the latter increasing immediately after the ingestion of tyrosine. Likewise, the response of the reducing substances to ascorbic acid in one patient (Case 1) was slower than that of the total hydroxyphenyl compounds. Despite the nonspecificity of the methods used the changes noted in the scorbutic patients would seem to be the result of the tyrosine and ascorbic acid administration since the regimes were constant except for these two variables.

The excretion of 'tyrosyl' derivatives by scorbutic patients who were maintained on a vitamin C free diet did not differ appreciably from that of normal subjects when they were not receiving tyrosine. The metabolic abnormality was noted only when they were given exogenous tyrosine. Furthermore the scorbutic patient (Case 3) who received only ascorbic acid and no tyrosine showed no statistically significant change in excretion of 'tyrosyl' derivatives after the administration of ascorbic acid. Normal Subject 3 demonstrated that the dietary regime had no significant role in 'tyrosyl' excretion.

The scorbutic patients demonstrated a progressive increase in capillary fragility while receiving tyrosine. While the death of the patient (Case 4) cannot be ascribed to a hemorrhagic lesion as a result of scurvy, nevertheless all the vitamin C deficient patients showed progression of the scorbutic skin lesions associated with increased weakness and lassitude during administration of L-tyrosine. Presumably the administration of amino acid in these large amounts placed an increased demand on the unmeasured body stores of vitamin C. In the original work of Scloock<sup>1</sup> it is implied that the feeding of tyrosine results in a depletion or excess utilization of the ascorbic acid of the body. This phenomenon was apparently demonstrated clinically under the conditions used in the present study by the progressive intensity of the signs and symptoms of scurvy. However in the normal individual there was no significant decrease in the ascorbic acid content of the whole blood during the administration of tyrosine and when these same individuals were given added ascorbic acid before and during administration of tyrosine it did not change their response to this amino acid.

That the abnormality of the metabolism of hydroxyphenyl compounds has wider implications than in scurvy alone has support in the effect that liver extract and pteroylglutamic acid have on the metabolism of hydroxyphenyl compounds. Swendsen and co-workers<sup>10, 11</sup> have reported that patients with pernicious anemia excrete abnormally large amounts of hydroxyphenyl compounds and that the excretion of these compounds decreases to normal levels when liver extract is given. Scloock and Lepow<sup>12</sup> also have shown that milder pernicious anemia extracts partially improved the tyrosine metabolism of scorbutic guinea pigs. Likewise pteroylglutamic acid has been shown to have an effect on the metabolism of hydroxyphenyl compounds in scorbutic guinea pigs both *in vivo*<sup>13</sup> and *in vitro* experiments<sup>14</sup>. In addition the administration of ascorbic acid to animals deficient in pteroylglutamic acid has altered the course of this deficiency.<sup>15</sup>

## SUMMARY

The metabolism of tyrosine was studied in four patients with scurvy and in three normal individuals. In six of these individuals, 20 Gm of tyrosine per day was given by mouth for periods varying from five to thirty one days.

The patients with scurvy, while on a vitamin C-free diet and oral administration of tyrosine, excreted large amounts of "tyrosyl" derivatives, and the ability of their urine to reduce phosphomolybdic acid increased markedly presumably due in part to the presence of *p*-hydroxyphenylpyruvic acid. The addition of ascorbic acid to this regime in the scorbutic patients resulted in a rapid decrease in the excretion of "tyrosyl" derivatives and the disappearance of the abnormal reducing material in the urine.

The excretion of "tyrosyl" derivatives in normal individuals on tyrosine was comparable with that of scorbutic subjects during the period when they were receiving ascorbic acid. At no time did the reducing substance of the urine of normal individuals rise significantly while they were receiving tyrosine. The addition of ascorbic acid to a normal individual's diet did not change the response to the administration of tyrosine.

It is concluded that individuals with scurvy have a marked defect in the metabolism of tyrosine and hydroxyphenyl compounds and that previous experiments with scorbutic animals and premature infants are in general agreement with the findings reported here.

It seems unlikely that this defective metabolism of tyrosine plays a significant role in the clinical picture of scurvy since the excretion of "tyrosyl" derivatives was not remarkable unless added tyrosine was given.

The clinical status of scurvy is made more severe during tyrosine therapy with increase in capillary fragility. These manifestations are completely controlled when ascorbic acid is added to the diet despite continued administration of tyrosine.

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# OBSERVATIONS ON THE ETIOLOGIC RELATIONSHIP OF ACHYLIA GASTRICA TO PERNICIOUS ANEMIA

## XI HEMATOPOIETIC ACTIVITY IN PERNICIOUS ANEMIA OF A BEEF MUSCLE EXTRACT CONTAINING FOOD (EXTRINSIC) FACTOR UPON INTRAVENOUS INJECTION WITHOUT CONTACT WITH GASTRIC (INTRINSIC) FACTOR

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PREVIOUS observations<sup>1, 2</sup> have shown that 200 Gm of beef muscle are hematopoietically active in addisonian pernicious anemia when 150 ml of normal human gastric juice also are given by mouth within six hours, or preferably simultaneously, as a daily regime for a period of ten days. If an acid mixture of beef muscle and gastric juice is incubated for twelve hours for the purpose of effecting peptic digestion of the beef muscle and is then neutralized, it also is active.<sup>3</sup> On the other hand, if the incubated mixture is heated to 100° C for five minutes, its hematopoietic activity as determined by oral administration is destroyed,<sup>3</sup> whereas that of whole liver or liver extract is not detectably affected by such a procedure. From this it was assumed that the thermostable antipernicious anemia principle of liver was *not* formed by the incubation procedure *in vitro* and that the effect of heat was merely to destroy a thermolabile factor in the gastric juice.

If a beef muscle and gastric juice mixture is given at an acid pH (1.8 to 3.5) subsequent to the twelve-hour acid incubation period, no hematopoietic effect appears.<sup>3</sup> It, however, this acid incubated mixture is treated with alkali to give a pH of 5 to 7 just prior to oral administration, it is active. This suggested that a preliminary chemical interaction occurred between the so called extrinsic factor of beef muscle and the intrinsic factor of normal human gastric juice at or about neutrality within the intestinal tract. Such a reaction was conceived as necessary for the eventual formation of the antipernicious anemia principle in the body. It seemed probable, therefore, that the extrinsic factor was chemically different from the antipernicious anemia principle of liver.

Because of this evidence, the increased hematopoietic activity in pernicious anemia of liver<sup>4</sup> and of relatively crude liver extracts<sup>5</sup> when given orally together with normal human gastric juice was originally assumed to indicate the presence of extrinsic factor as well as of the antipernicious anemia principle.<sup>6</sup> However, it was later shown<sup>7</sup> that even refined liver extracts when given by

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mouth were potentiated in their hematopoietic effect by gastric juice and that destruction of the antipernicious anemia principle of such extracts by hydrolysis with 5 per cent sulfuric acid resulted likewise in the loss of extrinsic factor activity. Recently it was found that the hematopoietic activity of orally administered pure vitamin  $B_{12}$  derived from liver (presumably the antipernicious anemia principle) also was enhanced by the simultaneous administration of normal human gastric juice—but that the activity of the combination when given orally was not so great as that of the vitamin  $B_{12}$  when administered alone parenterally.

The observations reported here concern questions obviously raised by these findings: (1) Can extrinsic factor *alone* (beef muscle) act directly as the antipernicious anemia principle—that is, is a suitable preparation of beef muscle, like vitamin  $B_{12}$ , hematopoietically effective in pernicious anemia upon parental administration without contact with gastric juice? (2) Is the hematopoietic potentiating action of intrinsic factor in pernicious anemia *specific* only for vitamin  $B_{12}$  and chemically similar substances in beef muscle and in other foods, or does intrinsic factor also facilitate the nonspecific absorption or otherwise enhance the hematopoietic action of other substances?

#### METHODS

All patients studied had Addisonian pernicious anemia either in relapse or without previous treatment. The diagnosis was established by the characteristic blood and bone marrow morphology and by the presence of histamine fast achlorhydria. It was confirmed in each patient by one or more reticulocyte responses to specific therapy and eventually by a return to normal blood values as a result of liver extract therapy. During the observations the basal diet of the patients contained no meat, fish, or eggs and little milk. The administration of the test substances, including the normal human gastric juice, was made at 8 P.M. The last meal of the day, consisting of tea and white toast, was at 4 P.M. This separated the test substance by at least four hours from any food constituent considered likely to contain extrinsic factor.

The attempted plan of the observations was to administer to the patients during three consecutive periods of ten or more days each a uniform daily dose of extrinsic factor derived from 400 Gm of beef muscle first orally, then orally with 150 ml of normal human gastric juice, and finally intravenously *without* gastric juice. In evaluating the hematopoietic activity of the various preparations used, the method of serial reticulocyte responses<sup>4</sup> was employed.

Formigne<sup>5</sup> has shown that a 70 per cent alcohol extract of beef muscle possesses extrinsic factor activity in pernicious anemia. Consequently the preparation of such an extract was undertaken, starting with a concentrated aqueous extract of lean beef muscle,\* of which 1 Gm was derived from 14 to 16 Gm of beef muscle. Following dilution of the aqueous extract with an equal volume of distilled water, a 70 per cent alcohol concentration by volume was made by the addition of suitable amounts of 95 per cent ethyl alcohol. The

\*Kindly supplied by Dr. William Peabody of the Valentine Company, Inc., Richmond, Va.

TABLE I. EFFECT OF DRUG

CASE PERIOD DAY OF THERAPY	10 ML MEAT EXTRACT* ORALLY						10 ML MEAT EXTRACT* AND 150 ML GASTRIC JUICE ORALLY							
	109		110		111		105		109		110		III	
	I		I		I		I		II		II		II	
	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	P.R.C. (MIL)	RET (%)
0	128	04	172	13	182	07	187	15	166	08		19		14
1		04		07		09		13		06		13		14
2		06		10	175	07	183	13		03		15		19
3		03	173	09		13		14	162	05	215	16	14 <sup>1</sup>	15
4	128	06		11		14	178	12		14		11		19
5	Tsf	07		14	151	12		13		13		13		20
6		07	196	19		08	191	23	135	14	185	20	140	25
7	159	06		34		09		38		10		25		32
8		07		21	158	08	190	52		12		17		32
9		12	224	26		10		59	159	16	253	17	15 <sup>1</sup>	32
10	166	08		19	Cont'd 111 II		211	68		29		17		41
11	Cont'd 109 II		Cont'd 110 II					72		23		15		41
12							249	37	150	15	Cont'd 110 III			42
13							53			18			154	32
14							258	23		23				32
15							End		140	33			Cont'd 111 III	
16										50				
17										80				
18									144	72				
19										74				
20										109				
21									199	79				
22										83				
23										91				
24									210	101				
25										71				
26										31				
										Cont'd 109 III				

Tsf Transfusion of 250 ml of packed red blood cells

\*10 ml of meat extract are derived from 100 Gm of lean beef muscle

†Rule indicates end of therapy in period

flocculent precipitate resulting was allowed to settle overnight at 5° C. The supernatant liquid was then filtered through a fluted paper filter and the precipitate washed on the paper with one-fifth the initial volume of 70 per cent alcohol. The precipitate was then discarded. Thereafter, the alcoholic filtrate was concentrated by free evaporation on electric hot plates. At the final point of concentration, the boiling point reached 108° ± 4° C. The volume of the concentrated filtrate was then so adjusted with distilled water that 1 ml was derived from 40 Gm of the original beef muscle. The filtrate was then bottled and autoclaved for twenty minutes. Throughout the preparation of the alcoholic extract, precautions were maintained to employ pyrogen-free fluids and apparatus.

When the preparation was given orally, 10 ml were diluted in either 150 ml of physiologic saline or in 150 ml of neutralized normal human gastric juice secreted after the injection of histamine. When given intravenously, 10 ml of the extract were mixed with 500 ml of 5 per cent sterile dextrose and water. The slow intravenous administration of the meat extract was followed in a few instances by moderate or mild febrile reactions. These reactions

## ADMINISTRATION OF VARIOUS SUBSTANCES

10 ml MEAT EXTRACT* INTRAVENOUSLY										1 µg VITAMIN B <sub>12</sub> INTRAMUSCULARLY					
106		107		108		109		110		111		108		111	
I		I		I		III		III		III		II		IV	
RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)	RET (%)	RBC (MIL)
18 02	2 07	09	1 79	03	2 10	00	—	15	—	22	1 55	56	1 79	12	—
04		08		02		42	2 32	21		17		50		18	
10 00		09	1 51	03		50		—	1 41	16		43		28	
00		08		02	2 15	29		13		12	1 67	31	2 23	32	
39 01		12		04		12	2 39	16		22		32		41	
04	2 17	20	1 44	06		18		25		26		42		49	
01 18		29		04	2 30	25		33	1 51	46	1 80	51	2 48	76	
40		31		06		44	2 58	39		55		63		46	
23 73		49		08		64		37		07		73		50	
83		46	1 51	13	2 63	48		26	1 60	59	2 00	53	2 58	48	
49 83		36		21		54	2 65	11		56		48		47	
64		25		28		27	End			78		30		39	
05 33	2 69	31		36	3 3	12	End		1 61	78		58	2 48	37	
26		38		47	End		0.5 µg of vitamin B <sub>12</sub> daily			63	2 13	36		20	
53 06		24	1 39	54	End					73		53	End		
End		18		59					1 63	71		53			
	3 01	13		57						60	2 31	52			
		16	1 55	56						54	End				
	3 09	20	Cont'd 108 II						17	42					
	End									30					
									174	34					
										21					
										14					
									179	12					
									Cont'd 111 IV						

usually developed with the first two or three injections and did not thereafter recur. No signs of subsequent sensitization to the extract or to beef appeared in any of the patients.

## OBSERVATIONS

*Is Extrinsic Factor Hematopoietically Effective Upon Parenteral Administration Without Intrinsic Factor?* The results of the observations upon the hematopoietic effect of the 70 per cent alcohol extract of beef muscle in seven patients with pernicious anemia are shown in Table I. Although the individual reticulocyte peaks are small, the trend of their values and the time of their maxima and of the clinical improvement exhibited by the patients convinced the observers of the validity of the conclusions drawn under the usual carefully controlled conditions of the tests. In one patient, Case 105, the extract derived from 600 Gm of beef muscle given orally daily together with 150 ml of normal human gastric juice was hematopoietically active. In two patients, Cases 109 and 111, the extract derived from 400 Gm of beef muscle was inert upon daily oral administration, was slightly active upon daily oral administration with 150 ml of normal human gastric juice, and was still more active upon daily intravenous administration without gastric juice. In one patient, Case 110, the preparation of extrinsic factor was weakly active upon

daily oral administration, was slightly more active when given with normal human gastric juice, and was still more active upon intravenous administration without gastric juice. Three patients, Cases 106, 107, and 108, received the extract without gastric juice by intravenous injection only. A definite reticulocyte response appeared in each instance. In Case 107 the red blood cell count

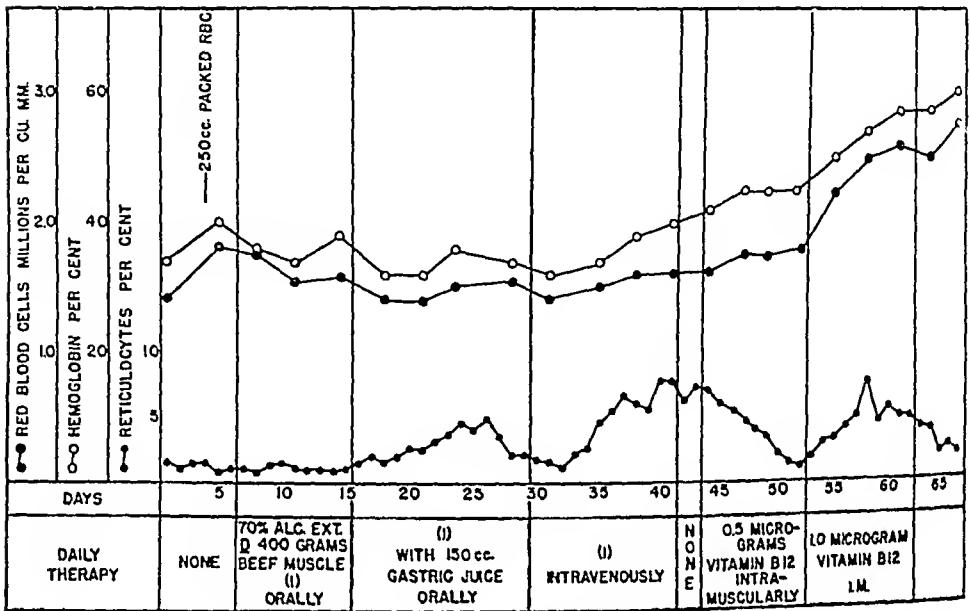


Fig. 1—Hematopoietic effects in Case 111 of the uniform daily administration of various substances in successive periods as shown. (I) refers to 10 ml. of 70 per cent alcohol soluble meat extract derived from 400 Gm. of beef muscle. Note the three successive reticulocyte peaks indicating respectively activation of the extract upon oral administration with normal human gastric juice, its greater activity upon intravenous administration without gastric juice and finally the still greater activity of Vitamin B<sub>12</sub> when given in a dose of 1 µg daily.

increased in twenty days from 2.07 to 3.09 millions per cubic millimeter. In two patients, Cases 108 and 111, the daily intramuscular administration of 1 µg of crystalline vitamin B<sub>12</sub>\* following the period of intravenous administration of the beef muscle extract resulted in another definite reticulocyte peak. From this it was inferred that the hematopoietic activity of the extrinsic factor preparation upon parenteral administration was distinctly less than that of 1 µg of vitamin B<sub>12</sub>. The results in Case 111 are shown in Fig. 1.

*Is the Hematopoietic Potentiating Action of Intrinsic Factor Specific?*  
In order to determine whether or not normal human gastric juice possesses a nonspecific effect in enhancing absorption from the alimentary tract in pernicious anemia, the blood levels of test substances presumably unrelated to vitamin B<sub>12</sub> were followed after oral administration to seven patients with pernicious anemia. Most of the observations were made before treatment, but a few were conducted subsequently during remission after specific therapy. Thus, blood glucose and blood tyrosine levels were determined following the

\*Kindly supplied by Dr. Augustus Gibson of Merck & Co. Inc., Rahway, N. J.

oral administration of single doses of 50 and 10 Gm respectively of those substances to fasting patients. The test substances were mixed together and given simultaneously with either 150 ml of normal human gastric juice or with an equal amount of physiologic salt solution upon alternate days. Blood alpha amino nitrogen and nonprotein nitrogen levels were similarly followed after the ingestion of 50 Gm of vitamin free casein (Labeo) (previously shown to contain no extrinsic factor)<sup>10</sup> with either physiologic saline or normal human gastric juice upon different days. Glucose determinations were made according to the method of Folin and Wu<sup>11</sup> and of Folin,<sup>12</sup> tyrosine by the method of Bernhart and Schneider<sup>13</sup> alpha amino nitrogen by the method of Krauel<sup>14</sup> and nonprotein nitrogen by the method of Folin and Wu.<sup>1</sup> As shown in Table II, no consistently enhanced absorptive effects upon glucose, tyrosine, or casein were observed that could be attributed to the gastric juice in any of the patients with pernicious anemia so tested before effective therapy. Not shown are the similar negative results obtained in a few patients after treatment.

The possible hematopoietic potentiating effect of normal human gastric juice upon synthetic pteroylglutamic acid\* either by increasing its absorption or by other action was also assayed in two patients. Cases 112 and 113, not included in the tables. To each 0.5 mg of pteroylglutamic acid was given orally daily during a first period of ten or more days together with 150 ml of physiologic saline, and during a second period of ten days the same amount of pteroylglutamic acid was given orally daily together with 150 ml of normal human gastric juice. In the first period of Case 112 a reticulocyte peak of 9.3 per cent was reached on the eighth day following an initial red blood cell count of 1.63 million per cubic millimeter. In Case 113 the corresponding value was 8.1 per cent reticulocytes on the eleventh day following an initial red blood cell level of 1.86 million per cubic millimeter. When gastric juice replaced the physiologic saline in the second periods no detectable reticulocyte peaks appeared in either patient. Thus no hematopoietic potentiating effect of normal human gastric juice upon pteroylglutamic acid was observed.

#### DISCUSSION

In 1938 Doek<sup>15</sup> wrote: "It seems probable that beef muscle contains the same material as liver but in quantities too small to be effective unless absorption is increased by gastric or enteric ferment. For some reason parenteral administration of muscle extract seems not to have been tried, but extract of muscle which had been incubated with gastric juice was potent in Wilkinson's experiments."

The potentiation of the hematopoietic activity of vitamin B<sub>12</sub> with gastric juice upon oral administration<sup>7, 8</sup> and the present confirmation of our preliminary observation<sup>7</sup> that the extrinsic factor of beef muscle is hematopoietically active upon parenteral administration *without gastric juice* go far to support Doek's surmise. It is of interest that Wilkinson<sup>16</sup> employed by daily intramuscular injection an extract derived from about the same amount of

\* Folvite kindly supplied by Dr Guy W. Clark of Lederle Laboratories Inc. Pearl River, N. Y.

TABLE II NBOATIVE L'FFECT OF NORMAL HUMAN GASTRIC JUICE IN AUGMENTING THE ADSORPTION OF ORALLY ADMINISTERED GLUCOSE,  
L TYROSINE, OR DIGESTION PRODUCTS OF VITAMIN FREE CASEIN

CASE	DATE	FOLLOWING 50 GM OF GLUCOSE BY MOUTH					FOLLOWING 10 GM OF L TYROSINE BY MOUTH				
		BLOOD GLUCOSE (MG PER 100 ML)					BLOOD TYROSIN (MG PER 100 ML)				
		FASTING	1 HR	2 HR	3 HR	4 HR	FASTING	1 HR	2 HR	3 HR	4 HR
107	2/7/49	87	163	174	117	89	Saline*	12	58	56	112
	2/8/49	117	248	271	186	125	N H G J †	11	42	61	51
	2/9/49	77	170	167	117	78	Saline				
	2/11/49	86	191	164	96	106	N H G J	16	81	96	87
108	12/15/48	131	133	140	131	120	Saline	13	32	35	45
	12/16/48	110	134	135	114	92	N H G J	21	47	69	74
	12/17/48	101	135	132	117	101	Saline	25	43	46	61
	12/20/48	125	118	143	144	134	N H G J	16	34	-	46
											71
109	3/1/49	85	-	88	107	87	Saline	14	21	6	35
	3/3/49	101	102	133	111	87	N H G J	14	26	38	43
110	3/1/49	64	85	86	66	63	Saline	12	29	44	46
	3/3/49	96	106	116	94	79	N H G J	15	29	39	47
111	2/2/49	114	138	143	133	136	Saline	11	30	44	52
	2/5/49	96	144	138	113	90	N H G J	14	30	10	41
	2/11/49	107	101	113	81	93	N H G J	15	44	65	64
											41
112	3/23/49	80	150	122	92	86	Saline	12	27	34	33
	3/28/49	-	89	145	126	75	N H G J	11	32	40	66
											43
											68
CASE	DATE	FOLLOWING 50 GM OF VITAMIN FREE CASEIN BY MOUTH					BLOOD ALPHA AMINO NITROGEN (MG PER 100 ML)				
		BLOOD NONPROTEIN NITROGEN (MG PER 100 ML)					VEHICLE (150 ML)				
		FASTING	1 HR	2 HR	3 HR	4 HR	FASTING	1 HR	2 HR	3 HR	4 HR
109	2/26/49	27.6	32.0	33.6	32.8	35.2	Saline	5.7	6.6	7.7	8.1
	2/25/49	26.8	24.4	34.4	33.2	37.2	N H G J	6.7	6.7	7.1	7.7
											7.6
											8.0

\*0.8% per cent solution of NaCl  
†Normal human gastric juice

beef muscle as we used. Although it was administered subsequent to incubation with an extract of hog mucosa, it seems probable that this was the first demonstration, though not then so interpreted, of the hematopoietic activity of a beef muscle extract upon parenteral administration.

The potentiating effect of normal human gastric juice upon orally administered vitamin  $B_{12}$  has been demonstrated.<sup>17</sup> The present observations fail to show any enhanced absorption by gastric juice of l-tyrosine, glucose, or of the digestive products of orally administered casein. Pteroylglutamic acid was not demonstrably more active in producing a reticulocyte response when given with gastric juice than with an equal amount of physiologic salt solution. On the basis of the present evidence then the action of intrinsic factor appears to be a specific one—presumably a hematopoietic potentiating effect on substances possessing identity with or chemical similarity to vitamin  $B_{12}$ .

There is suggestive evidence, however, that vitamin  $B_{12}$  may exist in nature, not necessarily in the pure form employed in these observations, but rather as protein 'conjugates' or analogues. Thus Smith and Parker<sup>18</sup> noted the "dramatic" increase in concentration effected by hydrolysis with trypsin of liver fractions containing vitamin  $B_{12}$ . According to Cohn and associates,<sup>20</sup> the antipernicious anemia activity of liver may remain largely with the proteins if their denaturation is avoided during the chemical fractionation of the liver. A synthetic modification of vitamin  $B_{12}$ , known as vitamin  $B_{12a}$ ,<sup>1</sup> with definite though reduced hematopoietic activity has recently been made by hydrogenation of vitamin  $B_{12}$ . By chromatography a second crystalline variant, designated as vitamin  $B_{12b}$ , has been prepared from liver and appears to possess biologic activity in both chick and microbial assays.<sup>2</sup>

Finally, from the comparative degrees of potentiation of pure vitamin  $B_{12}$  and of the present beef muscle extract by normal human gastric juice, at least an impression of a discrepancy is conveyed. Thus in our previously reported observations,<sup>7</sup> with the exception of Case 102 the potentiation of the activity of  $5 \mu g$  of pure vitamin  $B_{12}$  by gastric juice, though definite, was not remarkable in terms of reticulocyte responses. Yet in the present observations upon Cases 108 and 111, the daily dose 10 ml of the beef muscle extract employed, although possessing upon intravenous injection distinctly less than the hematopoietic activity of  $1 \mu g$  of pure vitamin  $B_{12}$ , was nevertheless capable of detectable increase in hematopoietic effect when given orally in the same dosage with gastric juice. According to microbial assays using the organism *Lactobacillus lactis* Dorner,\* the vitamin  $B_{12}$  content of 10 ml of this beef muscle extract was 0.9 microgram. Assay of the same extract using the organism *Lactobacillus leichmannii*<sup>21†</sup> demonstrated  $0.37 \mu g$  of vitamin  $B_{12}$  per 10 milliliters. In the latter method the effect of bacterial growth promoting substances other than vitamin  $B_{12}$  is stated to be determined by difference following destruction of the vitamin  $B_{12}$  in an aliquot portion of the sample by alkaline hydrolysis. This procedure completely destroyed the microbial activity for

\* These assays were kindly performed by the laboratories of Merck & Co. Inc., Rahway.

† The assays were kindly performed by Dr. Thomas H. Jukes of Lederle Laboratories Inc., Pearl River, N. Y.

*L. leuckmanni*, and so presumably indicates the absence of significant quantities of thymidine or of other desoxyribosides. The results obtained upon microbial assay are seemingly in reasonably close agreement with each other and with the clinical effects upon intravenous injection in Cases 108 and 111. However, as indicated, the potentiating effect of gastric juice upon the beef muscle extract when orally administered is perhaps greater than would be expected from less than 1  $\mu\text{g}$  of vitamin  $\text{B}_{12}$ . Thus, it is possible that substances in the beef muscle extract other than microbially active vitamin  $\text{B}_{12}$  may be effective as food (extrinsic) factor in pernicious anemia. Yet, so far as can be inferred from the negative results of the studies with glucose, *L*-tyrosine, casein, and pteroylglutamic acid, the function of the gastric (intrinsic) factor is a specific one.

#### SUMMARY AND CONCLUSIONS

1 Observations were made during successive periods of ten or more days in seven patients with Addisonian pernicious anemia employing a 70 per cent alcohol extract of beef muscle as a source of food (extrinsic) factor.

2 When 10 ml of the extract derived from 400 Gm of beef muscle were given daily by mouth to these patients, a detectable reticulocyte response appeared in only one instance. When the extract was given together with 150 ml of normal human gastric juice to four patients, reticulocyte responses appeared in all instances, and when the material was subsequently given intravenously without gastric juice to three of these patients, another reticulocyte response appeared, thus indicating greater hematopoietic activity.

3 In two patients it was shown that the hematopoietic effect of 10 ml of the beef muscle extract upon daily intravenous injection was less than that of the daily intramuscular injection of 1  $\mu\text{g}$  of crystalline vitamin  $\text{B}_{12}$ . Microbial assays indicated that 10 ml of the beef muscle extract contained from 0.37 to 0.9  $\mu\text{g}$  of vitamin  $\text{B}_{12}$  activity.

4 However, judging from our previous observations upon the potentiation of 5  $\mu\text{g}$  of crystalline vitamin  $\text{B}_{12}$  by normal human gastric juice, the hematopoietic activity of the beef muscle extract when given orally with gastric juice appeared to be surprisingly great.

5 Only this fact suggests that substances in the meat extract other than vitamin  $\text{B}_{12}$  are susceptible of potentiation by normal human gastric juice upon oral administration in pernicious anemia.

6 No evidence was obtained for a nonspecific effect of gastric (intrinsic) factor in increasing the hematopoietic action of pteroylglutamic acid or in promoting the intestinal absorption of glucose, *L*-tyrosine, or the digestion products of casein.

We desire to express our indebtedness to the late Dr. Lionel Berk, whose death unhappily interrupted the preliminary work that led to the present observations. We have received much valuable advice from Dr. Arnold B. Welch and Dr. Robert W. Henle of the School of Medicine of Western Reserve University. Finally, we are grateful to Miss Phyllis Gordon, Mrs. Katharine Bingham, and Miss Angela Pasquariello for the performance of the blood examinations.



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# FURTHER OBSERVATIONS ON THE USE OF THE URINARY PIGMENT-CREATININE RATIO FOR THE MEASUREMENT OF BASAL METABOLIC RATE

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**I**N A previous communication,\* it was found that an accurate correlation existed between the basal metabolic rates as determined by the respiratory calorimeter and the urinary pigment-creatinine ratio (P/C) † On the basis of statistically valid results obtained in 156 adult female subjects and 57 adult male subjects, regression equations were derived which permit the calculation of the basal metabolic rate from the urinary pigment-creatinine ratio These equations are

Male  
Female

$$\begin{aligned} \text{BMR} &= 57.0 + 0.25 \text{ P/C} \\ \text{BMR} &= 54.7 + 0.22 \text{ P/C} \end{aligned}$$

This present study is an analysis of the results obtained in comparing the basal metabolic rates determined by the respiratory calorimeter and the pigment-creatinine ratio in 740 observations made on 424 adult patients Of the 740 observations, 457 (61.7 per cent) were on euthyroid patients, 168 (22.8 per cent) were on hyperthyroid patients, and 115 (15.5 per cent) were on hypothyroid patients The clinical material consisted of hospital, private, and out-patient department patients

## RESULTS

We have previously reported\* that the determination of the pigment creatinine ratio is unreliable in the presence of increased bilirubinemia, azotemia, and in the presence of hemoglobinuria and drugs and foods which impart an abnormal color to the urine (e.g., riboflavin, aureomycin, beets, and rhubarb) There were 32 such cases in our series The pigment creatinine determination is also deficient in measuring hypometabolism, rarely indicating metabolic rates of less than -15 per cent There were 115 observations on cases of this type in our series Thus, there were 147 instances in which the pigment-creatinine determination would not be expected to be of diagnostic value

Of the remaining 593 observations, the BMR as determined by the respiratory calorimeter and the pigment-creatinine ratio agreed with each other and with the clinical findings in 508 cases (85.6 per cent) Of these 508 observations, 380 (64.0 per cent) were on euthyroid patients and 128 (21.6 per cent) were on hyperthyroid patients In 58 cases (9.8 per cent) the BMR as determined by the pigment-creatinine ratio was more consistent with the clinical findings than the BMR determined on the basis of oxygen con

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\*Vorzimer J J Cohen I B and Joskow J The Use of Urinary Pigment Excretion for the Measurement of Basal Metabolic Rate J LAB & CLIN MED 34 482 1949  
†The chemical determinations involved in this test can be done in about twenty five minutes and can be performed easily in any routine chemical laboratory

TABLE I RESULTS OF TESTS—EXCLUDING OBSERVATIONS IN WHICH P/C WOULD NOT BE USED FOR DIAGNOSTIC PURPOSES

RESULT	NUMBER OF OBSERVATIONS	PER CENT OF TOTAL
BMR and P/C agree with each other and with the clinical findings		
a Euthyroids	380	64.0
b Hyperthyroids	128	21.6
	508	85.6
P/C more accurate than BMR		
a Euthyroids	48	8.1
b Hyperthyroids	10	1.7
	58	9.8
BMR more accurate than P/C		
a Euthyroids	14	2.4
b Hyperthyroids	13	2.2
	27	4.6
Total	593	100.0

The P/C determination is not reliable in cases of jaundice, azotemia, and in the presence of hemoglobinuria or drugs that color the urine (3 cases) or in hypothyroidism—metabolism below -15 per cent (115 cases)

sumption. Of these 58 cases 48 (81 per cent) were euthyroid patients and 10 (17 per cent) were hyperthyroid patients. In 27 cases (4.6 per cent) the BMR determined by the pigment creatinine ratio was less in agreement with the clinical findings than was the BMR as determined by the respiratory calorimeter. Of these 27 cases, 14 (2.4 per cent) were euthyroid patients and 13 (2.2 per cent) were hyperthyroid patients. Thus in 566 observations (95.4 per cent), the BMR as determined by the pigment creatinine ratio in normal and hyperthyroid patients was of diagnostic value. In the remaining 27 cases (4.6 per cent) the pigment creatinine metabolic rate was inaccurate.

#### DISCUSSION

The observation that the BMR as calculated from the urinary pigment creatinine ratio is at least as accurate as that calculated from the respiratory calorimeter in 95.4 per cent of instances when cases in which the pigment creatinine ratio would not be expected to be accurate are eliminated indicates that the pigment creatinine BMR should be used to check the oxygen consumption BMR when the latter does not seem to agree with the clinical picture. In addition, there is a group (58 cases or 9.8 per cent of patients having metabolism tests) in which the BMR calculated by the pigment creatinine ratio should be used in preference to the BMR determined by oxygen consumption. We were able to break down this group into five types:

1. Cases of hyperthyroidism under prolonged therapy in which the BMR remains elevated in spite of clinical improvement—10 cases

*Example A.* E, a 48-year-old white man, was seen in the Outpatient department for the first time in December 1945, complaining of weakness, nervousness, palpitations, and weight loss for one year. Physical examination revealed an enlarged firm thyroid gland, warm moist skin, and a tachycardia. BMR was +30. He was given thiouracil and later changed to propylthiouracil in adequate dosage. The patient showed gain in weight and disappearance of all signs of thyrotoxicosis except that repeated BMR determinations remained between +30 and +48. The patient volunteered this information. When taking

a BMR I'm always nervous and feel as if I'm choking" In July, 1948, it was decided to stop all antithyroid medication as the patient was asymptomatic although the BMR was +30 In November, 1948, while the patient was still clinically euthyroid, the oxygen consumption BMR was +32 while the BMR calculated on the basis of urinary pigment creatinine ratio was successively -4 and +2 In January, 1949, while still asymptomatic, the oxygen consumption BMR was +36 while the BMR calculated from the P/C was -8

## 2 Cases in which an anxiety state or neuroendulatory asthenia causes an abnormally elevated BMR inconsistent with the clinical findings—32 cases

*Example A* S C, a 47 year old white woman, was admitted to the hospital March 3, 1949 She gave a three year history of nervousness, hypertension, palpitations, heat intolerance, and sweating In December, 1946, her BMR was +50 and a subtotal thyroidectomy was performed Her symptoms persisted for eighteen months postoperatively In July, 1948, she had a BMR of +7 with all the symptoms of thyrotoxicosis still present Her present admission was due to the appearance of a small nodule on the left side of the thyroid gland She showed no evidence of weight loss, there was no tremor, the pulse rate was 100 She was quite apprehensive about the prospect of further surgery On March 9, 1949, the oxygen consumption BMR was +24 whereas the P/C BMR was -11 On March 15, the oxygen consumption BMR was +21 while the P/C BMR was -13 On March 16, the patient was reassured that no surgery would be necessary She slept well that night and on the following day the oxygen consumption BMR was +7 and the P/C BMR was -8 The patient was discharged from the hospital with the diagnosis of menopause, essential hypertension, and anxiety state This patient had received no specific therapy at any time during her hospital stay

*Example B* G A, a 27 year old white woman, was admitted to the hospital in January, 1949, for an evaluation of an asymptomatic hypertension known to be present for two months The only significant finding on physical examination was a blood pressure of 200/140 On January 19, a BMR was reported as +34, the P/C BMR calculation was -7 On January 25, a repeat oxygen consumption BMR was -1, the P/C BMR at this time was -2 There was no specific therapy given during the hospital stay The patient was discharged with a diagnosis of essential hypertension

*Example C* E W, a 30 year old white woman, was referred to the private office of one of the authors (J J V) for the first time in April, 1949, because she had been found to have a BMR of +60 two weeks previously She complained of palpitations and a 22 lb weight loss in the past twenty months following a pregnancy Physical examination revealed a warm moist skin, thyroid moderately enlarged, pulse rate 128 per minute, and slight prominence of the eyes A BMR at this time was +47 The patient was started on cyclohexylmethylthiouacil, 300 mg per day With the institution of therapy, the patient began to feel better and gain weight, and the pulse rate fell although the BMR remained elevated Five weeks after she was first seen, she had no complaints, had gained 14½ lb, and the pulse rate was 72 per minute A BMR at this time was +33 Because of the discrepancy between the clinical condition of the patient and the oxygen consumption BMR, a P/C BMR was determined and found to be -3

## 3 Cases in which it is not technically possible to obtain an accurate BMR (e.g. edentulous patients, patients with punctured eardrums, an swallowers)—5 cases

*Example* G S, a 67 year old white woman, complained of nervousness There was no evidence of hyperthyroidism clinically, but because the patient was edentulous, numerous attempts to do a basal metabolism test were unsuccessful In October, 1948, two successive P/C BMR determinations were +3 and -2

## 4 Cases in which a basal metabolism test is done on a patient for the first time as part of a routine work-up and yields a high result where there is no evidence of hyperthyroidism—10 cases

*Example S F*, a 36 year old white woman came to the Outpatient department complaining of pains in the knees which were diagnosed as due to osteoarthritis. She was 58 inches tall and weighed 20½ pound. Aside from the obesity and arthritis the physical examination was negative. A routine oxygen consumption BMR was +20. The P/C BMR was +8.

5 Cases in which the patient refuses to undergo a metabolism determination—1 case

*Example I C*, a 22 year old white woman was known to be a diabetic for four years. She had had numerous hypoglycemic reaction. She was hospitalized because of frequent episodes of nervousness and sweating which her physician attributed to an anxiety state. She became very upset at the prospect of undergoing a basal metabolism test and refused it. A BMR calculated on the urinary pigment creatinine ratio was -3. This result confirmed her physician's impression.

#### SUMMARY

The results of the comparison of the basal metabolic rate as determined by the oxygen consumption method and by the urinary pigment creatinine ratio method (P/C) in 740 observations made on 424 patients are reported. Of the 740 observations 457 (61.7 per cent) were on euthyroid patients, 168 (22.8 per cent) were on hyperthyroid patients and 115 (15.5 per cent) were on hypothyroid patients. The clinical material consisted of hospital private and out patient department patients.

The BMR as determined by the pigment creatinine ratio (P/C) is unreliable in cases of hypothyroidism, hyperbilirubinemia, azotemia, and in cases in which the urine is discolored by drugs or foods. There were 147 such cases in this series. If these observations are eliminated then the P/C BMR is at least as accurate as the oxygen consumption BMR in 95.4 per cent of the remaining cases.

The P/C BMR should be used to check all cases in which the oxygen consumption BMR does not agree with the clinical findings in euthyroid and hyperthyroid patients.

The P/C BMR determination should be used in preference to the oxygen consumption BMR for the following groups of patients (9.8 per cent of our series):

- 1 Cases of hyperthyroidism under prolonged therapy in which the BMR remains elevated in spite of clinical improvement.
- 2 Cases in which anxiety state or neurocirculatory asthenia causes an abnormally elevated BMR inconsistent with the clinical diagnosis.
- 3 Cases in which it is technically impossible to obtain an accurate BMR.
- 4 Cases in which a basal metabolism test is done on a patient for the first time and yields a high result where there is no evidence of hyperthyroidism.
- 5 Cases in which the patient refuses to undergo a metabolism determination.

The authors appreciate the technical assistance rendered by Mrs Jennie Shotton, Miss Edna Arzt and Miss Virginia Rehmizter and are grateful to Mr Jules Joskow, Department of Economics, The City College of New York for his statistical analysis of the results.

## EFFECT OF RETAINED BRONCHIAL LIPIODOL ON BLOOD IODINE

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NUMEROUS studies have been made on the metabolism of iodine and blood iodine levels in normal people and in various pathologic conditions. Normal standards for protein-bound blood iodine have been established,<sup>1, 2, 3</sup> 30 to 85  $\mu\text{g}$  per 100 ml of blood being the quoted normal range. In our laboratory, 5 to 7  $\mu\text{g}$  per 100 ml is considered normal. Patients with pulmonary tuberculosis have normal protein-bound blood iodine values.<sup>4</sup> Normal blood iodine values have been seen in numerous other pathologic conditions. Most observers agree that hyperthyroidism produces an elevation of the protein-bound blood iodine.<sup>1, 2, 3</sup> Turner<sup>2</sup> found elevated blood iodine levels, up to 18  $\mu\text{g}$  per 100 ml of blood, in lymphatic leucemia. Several observers<sup>1, 6</sup> have found increased protein-bound blood iodine values in patients with liver and gall bladder disease. The effects of previously administered iodine containing compounds such as gall bladder dyes must be always borne in mind in these latter patients. It is known that the liver takes up iodine and excretes it into the bile. Cohn and Feldman<sup>5</sup> quote Greene and Bringer as finding normal blood iodine values in liver and gall bladder disease, if no iodine containing compounds had been taken previously. Cohn and Feldman<sup>5</sup> found experimentally that common bile duct ligation in cats failed to produce an elevation of blood iodine. The administration of gall bladder dyes, however, elevates the protein-bound blood iodine for several weeks. A group of patients with advanced liver disease was compared with a group of normal subjects, and blood iodine levels were found to be similar. Rabbits had no elevation of blood iodine following carbon tetrachloride poisoning to produce liver cell necrosis and extensive liver damage.<sup>5</sup>

It is quite clear that an elevated blood iodine may be due to hyperthyroidism and previous administration of iodine containing compounds, among other things.<sup>7, 8</sup> Chemical laboratories doing blood iodine determinations usually recommend that patients do not receive any iodine containing compounds for from ten to thirty days prior to the determination of this test.

No information can be found in the literature concerning the quantitative effect on the protein-bound blood iodine of retained Lipiodol in the bronchi and alveoli of the lung as a result of previous bronchograms. Means<sup>9</sup> has noted "the prolonged 'spurious' elevation of protein-bound iodine which follows the administration of such organic iodine containing compounds as

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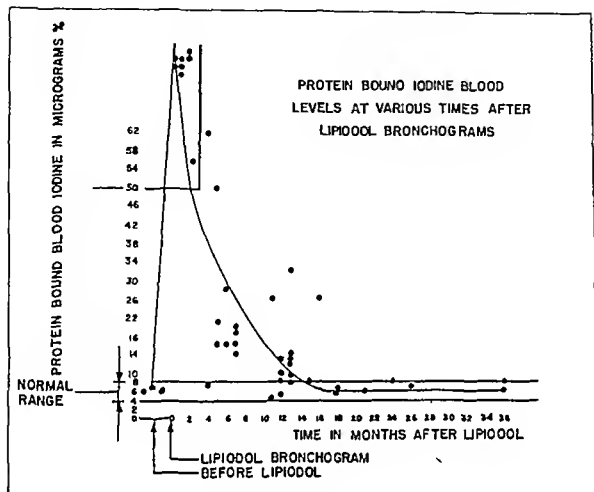
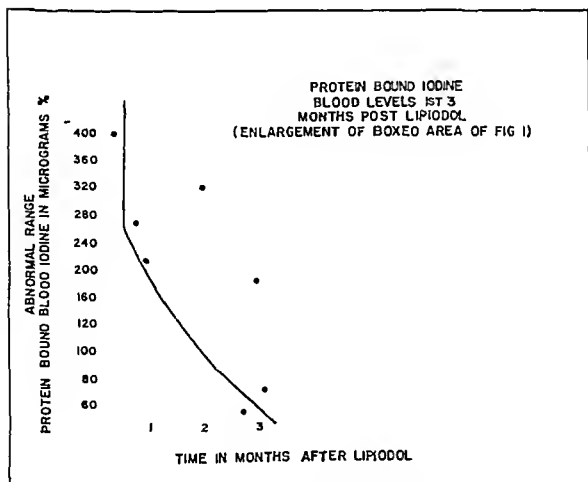


Fig 1



Fig

Iodikon or Lipiodol used in roentgenography" Salter<sup>10</sup> has stated that "the intrathecal injection of Lipiodol may form a localized reservoir of iodine which will slowly feed small amounts of iodine into the general circulation over the course of many weeks" Communication with several chemical laboratories and with the manufacturers of Lipiodol indicates that the quantitative effect

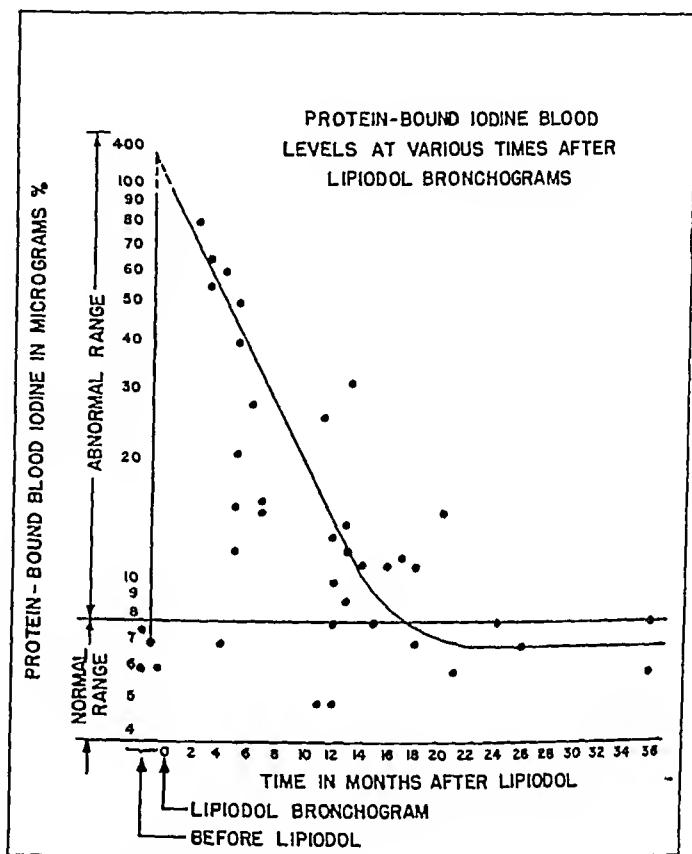


Fig 3

of retained Lipiodol is unknown and no studies have been published. The purpose of the present work is to determine the effect of retained Lipiodol upon the blood iodine.

Forty-one determinations of protein-bound blood iodine were secured on thirty patients at varying known intervals following the instillation of Lipiodol for a bronchogram. Four patients had normal protein bound blood iodine determinations before Lipiodol instillation (6, 6, 7, and 8  $\mu\text{g}$  per cent). It soon became apparent that retained Lipiodol causes an elevation of the blood iodine for prolonged periods of time. Values of over 400  $\mu\text{g}$  per 100 ml of blood were secured one week following the instillation of Lipiodol. The

\*Lipiodol 40 per cent containing 540 mg of iodine by weight per milliliter was used. Varying amounts from 5 to 20 ml were instilled for visualization of the bronchi.

† Blood iodine refers to protein bound blood iodine as determined by the method of Barker<sup>11</sup> modified by M. E. Morton using 10 per cent trichloroacetic acid to precipitate plasma protein.



protein bound blood iodine level dropped rather rapidly to average approximately 200  $\mu\text{g}$  per 100 ml of blood one month after instillation of Lipiodol (still greatly elevated) and then gradually dropped further for some time. It is important to note that the curve drawn of the various values of blood iodine plotted against the time since the last bronchogram did not reach the normal range (4 to 8  $\mu\text{g}$  per 100 ml blood) until seventeen months after the patient's last bronchogram (Figs 1, 2 and 3).

#### SUMMARY AND CONCLUSIONS

1 Instillation of Lipiodol for a bronchogram produces extremely high levels of protein bound blood iodine for prolonged periods (months).

2 It requires approximately seventeen months for the blood iodine level to reach normal in patients who have received bronchograms with Lipiodol. Individual patients may have elevated blood iodine values for three and four years.

3 Blood iodine cannot be used as a diagnostic agent (e.g. in suspected cases of hyperthyroidism) in patients who have had Lipiodol bronchograms within the previous two years and possibly longer.

Grateful acknowledgment is made to Ellen M. Bahr for the blood iodine determinations.

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# THE EFFECT OF THYROID SECRETORY ACTIVITY ON THE DISTRIBUTION OF RADIOIODINE IN PLASMA

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WHEN labelled inorganic iodide is administered by mouth, 90 per cent or more is absorbed in ninety minutes<sup>1</sup>. During this interval, and for several hours thereafter, a portion of the administered dose diffuses into the extracellular fluid. While absorption and diffusion are occurring and during the ensuing twenty-four to forty-eight hours almost all of the iodide ion is competitively removed from the blood and body fluid by the thyroid gland and the kidneys. That which is trapped by the thyroid is soon synthesized into hormone. A variable portion of the latter is stored in the gland while the remainder is secreted into the blood. Once thyroid hormone is released into the blood it apparently undergoes a slow degradation at the rate of about 5 per cent of the existing level per day<sup>2</sup>. Both the rate at which the thyroid gland removes iodine from the blood and the total amount ultimately retained by the gland are characteristically influenced by the state of thyroid activity provided the gland has not been previously saturated with iodine. Each has been measured repeatedly in human subjects by direct and indirect techniques<sup>3,4</sup>. However, a more positive measurement of thyroid function would necessitate an estimation of the actual rate of hormone secretion. This present study is, therefore, chiefly concerned with the problem of the rate of release of hormone and is based upon serial determinations of radioiodine in the organic fraction of plasma at successive intervals after the administration of a tracer dose of  $I^{131}$ .

## METHODS

Subjects selected for study included patients with either hyperthyroidism or myxedema, along with a group of euthyroid subjects to serve as controls. In none of the patients was the diagnosis equivocal because of grossly atypical clinical findings or inconsistent laboratory data. No subject had received iodine therapy within six weeks or antithyroid drugs within two weeks before the experiments were started.

The tracer dose of  $I^{131}$  was given orally and varied from 0.1 to 0.2 mc except for occasional patients with hyperthyroidism who received 0.4 millicurie. No normal subjects received more than 0.1 millicurie. The material was either carrier free or contained not more than 0.5 mg of NaI. Blood samples were drawn at intervals of two, eight, and twenty-four hours after ingestion of the tracer dose. These were oxalated and a 5 cc portion of plasma precipitated with 45 cc of Somogyi's zinc sulfate sodium hydroxide reagent (45 cc) as described by Man and associates<sup>5</sup>. After centrifugation, a 20 cc aliquot of supernatant was removed and evaporated to dryness in a 2 oz ointment tin (29 sq cm area) by the aid of an infrared lamp. The activity of this fraction was considered to represent that of inorganic iodine.

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TABLE I PLASMA LEVELS OF I<sup>131</sup> AS PER CENT OF TWO HOUR INORGANIC LEVEL

INDIVIDUAL VALUES		PATIENT	HYPERTHYROIDISM	L	E	L	B	J	P	E	A	E	J	E	M	W	P	A	P	I	B	L	P	E	F	I	K	MEAN VALUES	
HYPERTHYROIDISM																													
Inorganic fraction	2 hr 8 hr 24 hr Slope 2.8 hr	100 140 0 -14	34 0 -16	- 37 -	460 200 -9	240 0.5 -13	49 20 16	30 39 16	110 0 15	170 13 14	80 52 82	110 84 15	72 33 16	420 70 10	42 0.6 16	100 157 9 ±0.69†													
Organic fraction	2 hr 8 hr 24 hr Slope 2.4 hr	100 140 62 +0.13	250 12 40 +0.17	40 180 -	15 0.3 0.0 -0.02	25 29 27 -0.01	08 19 54 +0.22	120 50 210 +0.22	330 490 30 +1.5	80 52 82 +0.19	110 84 15 +0.59	72 33 16 +1.00	420 70 10 +0.08	42 0.6 16 +0.09	100 157 9 ±0.14														
NORMAL SUBJECTS																													
MYXEDEMA																													
Inorganic fraction	2 hr 8 hr 24 hr Slope 2.8 hr	100 66 19 -6	30 10 -7	47 12 -9	64 -5 -1	70 17 -4	69 34 -1	71 12 -5	69 8 -3	31 4 11	43 8 -1	37 3 10	572 163 -7 ±0.69																
Organic fraction	2 hr 8 hr 24 hr Slope 2.4 hr	100 66 17 -0.03	24 14 0 -0.03	10 0.4 -0.03	12 13 0.8 -0.06	86 34 51 +0.11	30 110 37 -0.46	200 - 12 -	93 110 52 -0.36	3 13 0.7 -0.04	0.6 0.3 0.1 -0.01	150 97 31 -0.29	82 44 22 -0.13 ±0.03																
Inorganic fraction	2 hr 8 hr 24 hr Slope 2.8 hr	100 68 27 -53	30 20 -72	96 76 -0.7	60 22 -67	103 96 0.0							100 77 48 -1.0 ±1.5																
Organic fraction	2 hr 8 hr 24 hr Slope 2.4 hr	100 68 30 -0.08	58 30 16 -0.09	66 72 88 +0.10	38 23 0.9 -0.09	10 16 12 -0.02							47 38 32 -0.036 ±0.036																

Nodular glands (all others are diffuse)

†Standard error of mean

Nodular glands (all others are diffuse)

†Standard error of mean

The protein precipitate which contained organic hormonal iodine was washed three times with 50 cc portions of water. Assays of the washings on six different blood samples indicated that any additional washing would not remove more activity than 0.5 per cent of that in the total supernatant fraction from which the precipitate was removed. The washed precipitate was quantitatively transferred to a weighed ointment tin and dried under the heat lamp. It was then distributed evenly over the bottom of the tin with the help of a stirring rod and a small quantity of acetone. The half density of the precipitate in the containers weighed only 10 mg per square centimeter.

Counts were made under a Geiger Muller tube with a 6 mg per square centimeter mica window, 6.2 cm in diameter and connected with a scale of 8 counter. An appropriately sized aliquot of the original material was counted at the same time so that decay corrections were unnecessary. Aliquots of the standard and organic fraction were combined with 1 cc of a solution recommended by the Oak Ridge National Laboratory to prevent loss of volatile iodine.\*

### RESULTS

Individual values are recorded in Table I, and composite curves of the inorganic and organic fractions are depicted in Fig 1. The value of the inorganic fraction at two hours was arbitrarily set at 100 per cent as reference for the remaining determinations. Since total blood or body iodine content was not known, there was no advantage in employing the original dose as a standard for comparison.

The inorganic fraction is seen to decline much more rapidly in hyperthyroid patients than in normals. This is presumed to be due to the more rapid rate of removal by the thyroid gland, provided there is no significant increase in the rate of renal removal. It is of interest that the decrease of plasma iodine with time in the three curves does not give a straight line of constant slope on a semilogarithmic plot. Without more points on the curve it is impossible to come to any exact conclusions, but it may well be that this reflects the sequential operation of several biologic processes, each in itself expressed by a true exponential function.

A more rapid decline is present between the second and eighth hour than between the eighth and twenty-fourth. The initial rapid fall could be due to two factors: (1) The passage of iodine into the extracellular fluid in addition to the thyroid and the urine. (2) An exaggerated peak of the blood concentration if the rate of absorption should materially exceed the rate of diffusion into the extracellular fluid. The slope of the three curves between the second and eighth hour when inorganic iodine is being disposed of most rapidly may be seen in the table and in Fig 1. The difference between hyperthyroid and normal subjects is very real. However, the five patients with myxedema, although showing a flatter average curve, do not differ from the normals to a statistically significant degree.

The behavior of the organic fraction likewise is not the same in hyperthyroid subjects as in normals. Between the eighth and twenty-fourth hour the patients with hyperthyroidism show a distinct rising trend, while the curve of the normal subjects tends to decline. This difference in behavior of the two curves is a real one, as an analysis of the slopes of the two groups

\*NaHSO<sub>3</sub> 500 mg    Nil 250 mg    NaOH 750 mg    water to 1 000 cc

will reveal (Table I). The trend of the organic fraction in myxedematous patients is not significantly different from that of the normal group. An observation to be discussed later is the high activity which was encountered in the organic fraction at the two hour period.

#### MEAN PLASMA RADIOACTIVE IODINE LEVELS

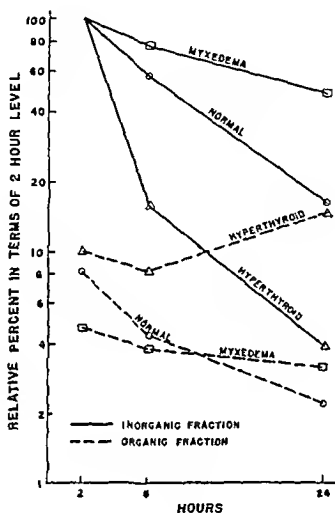


Fig. 1

In four of the hyperthyroid patients and four normals additional measurements were made after forty eight hours. The trend of the organic and inorganic curves during this interval was similar to that observed between the eighth and twenty fourth hour.

#### DISCUSSION

McConahey, Keating, and Power have recently reported their results of blood fractionation studies in a small series of patients and normal subjects who were given doses of  $I^{131}$  ranging from 1 to 100 millicuries<sup>8</sup>. Our own findings are similar in some respects to those reported by this group of workers. The chief point of difference is that the organic fraction in their three normal patients and also in the one hypothyroid patient showed a progressive rise albeit with a more gradual slope than in the series of hyperthyroid cases. The curves for both normal and hypothyroid patients presented here show a slight progressive fall in activity of this fraction. The reason for

this decline cannot be determined with any degree of precision, however, certain general principles should be noted. There is a slow process of disintegration of thyroid hormone in the body fluids which should proceed at approximately equivalent rates in all subjects. In hyperthyroidism, with relatively little hormone stored in the gland and a relative high uptake of radioiodine, it is to be expected that the material secreted after a tracer dose would be proportionately rich in radioactive iodine. On the other hand, with a normal gland, the comparatively large amount of preformed, nonradioactive hormone would force the gland to deliver to the blood a product relatively poor in the newly acquired radioactive component. Thus it is conceivable that in its normal state of activity the quota of radioactive hormone delivered to the blood would be insufficient to result in a steadily mounting activity within the organic fraction. The rate of change of organic iodine in the blood, therefore, does not offer a direct measure of the secretory activity. That the behavior of the organic fraction is quantitatively influenced by thyroid hyperactivity is nevertheless clearly demonstrated by the difference in slope of the curves as shown here.

The finding of small but unmistakably real quantities of active material in the organic fraction of patients with myxedema in the present study, and in that of McConahey and co-workers, is of interest. Moreover, our own finding of a relatively high value as soon as two hours after ingestion with a decline during the next six hours even in the presence of hyperthyroidism should also be commented upon. An explanation of the presence of active material in myxedematous patients, along with the temporarily high level in all patients, might suggest an extrathyroidal synthesis of an organic iodine complex. Incubation of serum with inorganic iodine under physiologic conditions has not been found to promote such a synthesis.<sup>6</sup> Morton and co-workers, on the other hand, have demonstrated the *in vivo* production of thyroxine and diiodothyroxine in thyroidectomized rats as early as two hours after the administration of labelled inorganic iodide.<sup>7</sup> It is difficult to believe, however, that the high level of serum activity here encountered at two hours could be achieved so rapidly by extrathyroidal synthesis. Moreover, the initial drop in the curve (between two and eight hours) is too rapid to be attributed to simple thyroxine decay. There is a possibility that a portion of the protein-bound material is not actually a hormonal compound but that it is fixed to protein fully as firmly and resists removal by washing.

The usefulness of a determination of blood radioiodine as a diagnostic test cannot be determined on the basis of the present observations. Significant differences between mean values can easily be demonstrated, but an overlap exists. The steepest slope for the curves of inorganic iodine (two to eight hours) in the normal group was -11 (Table I). However, two hyperthyroid patients (E N and E F1) showed slopes which were less steep. Both patients suffered from a relatively chronic type of disease of only moderate severity and each had a rather large nodular gland. In the case of the organic fraction, if one arbitrarily divides hyperthyroid patients from normals on the basis

of a positive or negative slope between eight and twenty four hours, the overlap consists of one normal patient with a positive slope and two hyperthyroid patients with slightly negative slopes

Thus the large majority of our cases show definite differences between normal and hyperthyroid blood values. Just how much overlap actually exists can be shown only by a large series. It may well be that this type of determination will prove a useful adjunct to already existing tests for hyperthyroidism.

Freedberg and co-workers have recently reported that protein bound radioiodine twenty four hours after a tracer dose was consistently higher in hyperthyroid patients than in normal controls.<sup>8</sup> In the present study the rate of change between eight and twenty four hours correlated with clinical status more consistently than did the final twenty four hour level.

The behavior of the inorganic fraction in myxedema was not greatly different from normal and the rate of change of the organic component was not distinguishable from that in the normal group. In our own experience the best diagnostic criterion for myxedema when one employs radioiodine techniques is the failure to demonstrate any local uptake of activity by direct measurement over the gland with a counter.

#### SUMMARY

After a tracer dose of radioiodine the activity of the inorganic fraction of the blood in hyperthyroidism declines more rapidly than normal while in myxedema the decline tends to be somewhat slower than normal. The activity of the organic fraction is distinctly elevated in all types of cases at the end of two hours, and then tends to decline between the second and eighth hour. This decline is progressive for the ensuing sixteen hours in both normal subjects and patients with myxedema; however, in patients suffering from hyperthyroidism there is characteristically a progressive rise in this fraction between the eighth and twenty fourth hour.

We are indebted to Ethel Buchwald Chudack for technical assistance.

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## INHIBITION OF THE GROWTH OF STAPHYLOCOCCUS AUREUS BY HUMAN SEMEN

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THE presence of penicillin in blister fluid of human skin following parenteral and topical application was determined by Dostrovsky, Gurevitch, and Rozansky.<sup>1</sup> In another study the concentration of penicillin in human milk was determined by Rozansky and Brzezinsky.<sup>2</sup> In pursuing these studies further we attempted to determine whether penicillin is excreted in the human semen. It was first necessary to find out whether semen in itself has any inhibitory effect on the growth of bacteria. Preliminary examinations revealed inhibition of the growth of *Staphylococcus aureus* by human semen. The following is a detailed report on a study of the inhibition of the growth of *Staph aureus* by human semen.

### MATERIALS AND METHODS

Twenty-eight specimens of semen obtained from twenty-four men between the ages of 27 and 47 years were examined. The semen of four patients was examined twice. Five of the examined specimens were azoospermic, ten were oligospermic (spermatozoa count lower than 60,000,000 per cubic centimeter), and thirteen were normospermic. The patients applied to the clinic for consultation in connection with sterile marriage. Only one patient (Specimen 21) had received 60 mg. testosterone propionate (six injections of 10 mg.) before examination. All the others had received no previous treatment. In most instances the semen was obtained under as sterile conditions as possible by masturbation in the outpatient clinic, but in four instances specimens were brought to the clinic from outside. Each specimen was tested for bacterial contamination before examination. One specimen was discarded because of contamination by *Esch. coli*. In five other ejaculates a few colonies of *Staphylococcus albus* were found.

The inhibitory effect of human semen on bacterial growth was examined by the cup method used in studies on the concentration of antibiotics.<sup>3</sup> The first test strain was *Staph. aureus*, Heatley, in use in antibiotic studies in this laboratory for a period of four years. In the course of later experiments two additional strains of *Staph. aureus* freshly isolated in the laboratory were used: strain 924 isolated from the urine of a patient with cystopyelitis, and strain 598 isolated from the pus in a case of furunculosis. All three strains had the usual properties of pathogenic strains of *Staph. aureus*: coagulase positive, marmite positive, actively hemolytic.

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Examinations were conducted as follows. One cubic centimeter of a 1:1,000 dilution of a twenty-four hour broth culture of the test strain was added to 20 c.c. of nutrient agar (Difco) and plates were poured. The plates were left at room temperature for four hours after which five cups 8 mm. in diameter (external) were placed on each plate. Four cups each received 3 drops of the tested ejaculates and the fifth received 3 drops of a penicillin solution containing 0.1 unit per cubic centimeter. The cup containing penicillin served as a control of the area of inhibition of growth of the staphylococci. The results were read after twenty-four hours of incubation at 37° C. In most examinations two plates were prepared for each specimen examined. The specimens were left at room temperature for four hours before examination to permit the ejaculates to liquify and to become homogenous. When sufficient material was available residues were stored at 8° C. after the first examination and were re-examined twenty-four hours to seventy-two hours later.

#### RESULTS

The results of the examinations are summarized in Tables I, II, and III, comprising studies in the Heatley strain strain 924 and strain 598 respectively.

As may be seen in Table I twenty-three of the twenty-eight specimens studied with the Heatley strain inhibited the growth of the staphylococci over an area from 10 to 20 mm. in diameter while five gave negative results. The positive results include examinations repeated after twenty-four and seventy-two hours. In three instances no inhibition was found at the first examination but the same specimens were positive when examined twenty-four and seventy-two hours later. Of the twenty-three positive specimens fourteen yielded similar areas of inhibition in both plates and nine specimens yielded positive results in one plate (four of these specimens were examined with one plate only). Specimen 17 was examined in three plates and areas of inhibition 16 mm. in diameter were observed in all of them. In six instances the semen was re-examined following twenty-four hours of storage at 8° C. Five of these specimens were positive and one was negative. Three of the positive specimens were particularly interesting because they were negative on first examination but yielded a distinct area of inhibition twenty-four and seventy-two hours later. Following two weeks of storage at 8° C. Specimen 14 yielded an area of inhibition similar to that of its first examination. Specimen 21 was negative following one week's storage at 8° C.

The inhibitory effects of eighteen specimens of human sperm on *Staph. aureus* strain 924 isolated from mine are summarized in Table II.

Fourteen of the eighteen specimens were positive five in two plates and nine in one plate. Four specimens in this series were negative.

The inhibition of *Staph. aureus* strain 598 isolated from a furuncle, by eight specimens of human sperm is summarized in Table III. Of the eight examinations five were positive each of them in two plates and three were negative when examined in one plate only.

TABLE I INHIBITION OF GROWTH OF STAPH AUREUS, HEATLEY STRAIN, BY HUMAN SEMEN

SPECIMEN	AGE OF PATIENT	QUANTITY OF SEMEN (C C)	SPERMATOZOA (MILLIONS/C C)	ZONE OF INHIBITION IN MM AFTER				
				4 HR		24 HR		72 HR
				PLATE I	PLATE II	PLATE I	PLATE I	PLATE II
1	27	1	Azoospermia	17	-	-	-	-
2	25	2	80	14	14	-	-	-
3	30	3	2	19	19	-	-	-
4	29	4	70	11	0	-	-	-
5	32	3	120	11	0	-	-	-
6	38	1	85	14	-	-	-	-
7	29	3	40	10 5	0	-	-	-
8	37	4	30	14	0	13	-	-
9	44	3	6	0	0	12	-	-
10	47	2 5	130	0	0	-	-	-
11	39	8	1	0	-	0	0	0
12	33	3	Azoospermia	0	0	15	12	11
13	27	6	70	0	0	15	15	15
14	39	4	40	14	10	-	-	-
15	26	3	20	0	0	-	-	-
16	28	3	70	0	-	-	-	-
17	30	2	Azoospermia	16	16	-	-	-
18	38	3	60	10	10	-	-	-
19								
(1, on second examination)	27	1	Azoospermia	0	0	-	-	-
20								
(17, on second examination)	30	1 5	Azoospermia	16	15	-	-	-
21	44	2	50	16	15	-	-	-
22	32	3	45	15	12	-	-	-
23	35	3	70	11	0	-	-	-
24	42	4	35	16	15	-	-	-
25	41	3	108	17	16	-	-	-
26	35	3	80	13	-	-	-	-
27								
(6, on second examination)	38	2	85	11	9	15	-	-
28								
(18, on second examination)	38	5	65	20	15	-	-	-

- Not examined

0 No inhibition

TABLE II INHIBITION OF GROWTH OF STAPH AUREUS, STRAIN 924, BY HUMAN SEMEN

SPECIMEN	ZONE OF INHIBITION IN MM AFTER 4 HR	
	PLATE I	PLATE II
10	11	-
11	18	-
12	10	-
13	11	-
14	15	10
15	0	-
16	0	-
17	14	14
18	0	-
19	0	15
20	20	12
21	12	0
22	11	-
23	15	0
24	12	13
25	14	-
26	10	0
28	15	-

- Not examined

0 No inhibition

TABLE III INHIBITION OF GROWTH OF *STAPH. AUREUS*, STRAIN 598, BY HUMAN SEMEN

SPECIMEN	ZONE OF INHIBITION IN MM. AFTER 4 HR	
	PLATE I	PLATE II
14	12	11
16	17	17
17	12	12
20	14	11
21	12	12
22	0	-
23	0	-
28	0	-

- Not examined

0 No inhibition

## COMMENT

Human semen has been found capable of inhibiting growth of several strains of *Staph aureus*. Inhibition was observed in 80 per cent of the examinations done with three strains of *Staph aureus*. The active principle of the semen was not associated with the spermatozoa, since only the supernatant fluid of the semen was used in the experiments. This is supported by the fact that four of the azoospermic specimens showed inhibitory activity. At this stage of the investigation little can be said as to the nature of the active principle. The factor is stable when stored for days at 8° C. Specimen 14 remained active even after fourteen days. The active principle is thermostable. Specimen 18 remained positive after heating for half an hour at 56° C. Specimens 24 and 25 retained their activity after exposure to 75° C for fifteen minutes.

Three specimens were negative in the first examination but found to be positive in examinations repeated after twenty four and seventy two hours. It may be of interest that these three specimens were not kept at room temperature as is usual in these experiments, but were placed mistakenly in the refrigerator when brought from the outpatient clinic. It may be assumed that the homogenization of these specimens, which takes place at room temperature was interfered with by refrigeration.

Six specimens of sperm which inhibited *Staph aureus* had no effect on the growth of *Fsch coli*.

## SUMMARY

Twenty eight specimens of human semen were tested for their inhibitory effects on the growth of *Staph aureus*. Eighty per cent of the specimens inhibited the growth of this organism. Preliminary studies indicate that the active principle is associated with the liquid part of the semen. Some properties of the active principle in the semen are discussed.

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# OBSERVATIONS ON THE USE OF A NEW ANALGESIC, NU 2206 (3-HYDROXY-N-METHYLMORPHINAN HYDROBROMIDE)

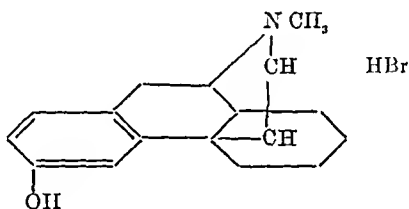
L L ZAGER, M D, W W SAWTELLE, B A, M D, E G GROSS, B S, M S, M D,  
P H D, S F NAGAYA, B A, M D, AND R T TIDRICK, B A, M D  
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## INTRODUCTION

THIS is a preliminary report on a clinical study of the analgesic properties of Nu 2206\* (3-hydroxy-N-methylmorphinan-hydrobromide) which includes observations on a series of normal subjects, on a series of patients with postoperative pain, and on a series of patients with intractable pain due to cancer. In instances abstracted in this report it was possible to observe the effect of this drug during and after prolonged use.

## CHEMISTRY

Nu 2206 (3-hydroxy-N-methylmorphinan-hydrobromide) has the following structural formula<sup>1</sup>



Nu 2206 occurs in the form of colorless crystals melting at 193° to 195° C. The substance is rather soluble in water at 20° C. It is easily soluble in water at 100° C. and in alcohol, but it is insoluble in ether. It has analgesic properties similar to those of morphine and some of the other morphine-like compounds developed recently.<sup>2,3</sup>

## ADMINISTRATION TO NORMAL SUBJECTS

The effect on the pain threshold in six normal volunteer student subjects was determined by the Hardy-Wolff-Goodell technique. Doses used were 0.5, 1.0, 2.0, and 3.0 milligrams. Each student received two subcutaneous doses of each of the four graded doses. No subject received more than two doses each week. These doses were compared with doses of 5.0, 10.0, and 15.0 mg of morphine sulfate given in similar manner and under similar conditions. The results indicated that Nu-2206 was about four times as potent as morphine on a per milligram basis. Peak threshold effects were reached at about the same time as with morphine but there was a somewhat greater duration of effect. The blood pressure changes were no greater than could be accounted for by sedation and resting of the subject.

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\*Supplied by Dr M J Schiffman, Hoffmann-La Roche Inc, Nutley, N J

Doses of 0.5 to 1.0 m<sub>g</sub> apparently produced no symptoms in any of the volunteers other than awareness of some vague gastrointestinal distress. Slight sedation may have occurred in some instances. Doses of 2.0 m<sub>g</sub> produced some nausea (but no vomiting), dizziness and sedation. The subjects were aware of gastrointestinal contraction and some inability to concentrate. Doses of 3.0 m<sub>g</sub> produced some nausea in practically all subjects, vomiting occurred in two different subjects on one occasion each and dizziness occurred in all subjects. Sedation, inability to concentrate and awareness of gastrointestinal symptoms were experienced by all subjects. In one subject vomiting occurred five to six hours after the injection. No euphoria was experienced by any of the subjects. In general 3.0 mg of Nu 2206 produced fewer and less severe symptoms than an equivalent analgesic dose of morphine. All subjects on 3.0 doses stated they either went to bed right after dinner (five to six hours after injection) or were too sedated to do any studying during the course of the evening. Respiration was not studied but by casual observation no significant respiratory changes were noticeable.

#### NU 2206 IN THE TREATMENT OF POSTOPERATIVE PAIN

Nu 2206 was used subcutaneously in a total of fifty-eight postoperative urological and general surgical patients. Thirty-six patients (twenty-nine urological and 7 general surgical) were given 1.5 milligrams. Twelve urological patients were given 4.5 milligrams. Six general surgical patients were given

TABLE I COMPARISON OF ANALGESIC POTENCIES FOLLOWING SUBCUTANEOUS INJECTION

DRUG	DOSE (MG)	NUMBER OF DOSES	HIGHEST % RISE THRESHOLD $M \pm \sigma$	TIME TO REACH PEAK $M \pm \sigma$ (MIN)	DURATION TO 10% OF PEAK $M \pm \sigma^*$
Morphine SO	10	1	21.5 $\pm$ 1.9	73.1 $\pm$ 8	246 $\pm$ 51
Morphine SO	15	1	21.2 $\pm$ 2.3	84.0 $\pm$ 7.7	280 $\pm$ 7.4
Nu 2206	1	11	10.1 $\pm$ 3.0	65.0 $\pm$ 9.3	211 $\pm$ 13.9
Nu 2206	2	9	18.2 $\pm$ 2.1	82.0 $\pm$ 5.9	267.0 $\pm$ 21.4
Nu 2206	2	9	24.8 $\pm$ 2.9	91.1 $\pm$ 8.3	301 $\pm$ 27.2

$M \pm \sigma$  is the mean effect + its standard deviation

3.0 milligrams. From general surgical patients were given 6 milligrams. The majority of the patients to whom 1.5 m<sub>g</sub> were given had transurethral resections. These patients usually have very definite discomfort and in some cases severe pain. Ordinarily these patients are given 10 m<sub>g</sub> of morphine sulfate immediately after completion of the procedure as the spinal anesthesia is wearing off and the same dosage is usually repeated in several hours. In these patients Nu 2206 in a dosage of 1.5 mg was uniformly ineffective. In the patients who had other miscellaneous urologic procedures—three nephrectomies, two suprapubic cystotomies and one orchidopexy—there was also failure to relieve pain. The one good result in urological patients was in a 9-year-old boy who had a nephrectomy. In the general surgical patients in this dosage range four obtained relief and three obtained no relief. These seven patients represented a variety of operations. It may be that these surgical procedures did not result

in as much postoperative pain as the average urologic procedure. It appeared that 15 mg of Nu-2206 was an unsatisfactory dose.

In six instances Nu-2206 was used in dosage of 30 mg for treatment of postoperative pain in general surgical patients. It afforded relief in three instances and gave insufficient relief in the remaining three. One of the latter patients subsequently obtained relief of pain with 6 milligrams.

Eleven urological patients who had had transurethral resection were given fifty one 45 mg doses. However, in this series, Nu-2206 was not given unless the patient had real pain and requested relief. In this dosage range, which was felt to be roughly equivalent to 15 mg of morphine, Nu-2206 was uniformly effective in relieving pain. Demonstrable effect was noted in most instances thirty minutes after administration of the drug. Analgesic effect was present longer than that observed with morphine and in most instances it was effective for six hours or more. There were no undesirable side effects.

Six milligram dosage was employed in four general surgical patients. In all of the patients to whom 60 mg were given, excellent relief was obtained. The following detailed case is presented as an example of this series.

Patient M W (48-9796), a 25-year-old man, was admitted on Aug 8, 1948, following multiple injuries received in an auto racing accident. Among his injuries were a perforating wound of the abdominal wall, peritonitis, traumatic rupture of the spleen, severe contusion of the colon, fractures of the transverse processes of the third and fourth lumbar vertebrae, and fracture of left zygoma. Surgical procedures included a splenectomy and colostomy.

TABLE II PATIENT M W

DATE	TIME	DRUG	REMARKS
8/31/48		100 mg Demerol 5 times per day	Relief
9/ 1/48		100 mg Demerol 5 times per day	Relief
9/ 2/48		100 mg Demerol 5 times per day	Relief
9/ 3/48	0300	3 mg Nu 2206	No relief
	1045	1 mg Nu 2206	No relief
	1630	3 mg Nu 2206	No relief
	2000	3 mg Nu 2206	No relief, emesis, excessive perspiration
9/ 4/48	0215	3 mg Nu 2206	Slept for more than 3 hr
	1700	3 mg Nu 2206	Relief from pain for about 3 hr
	2230	3 mg Nu 2206	Good relief
			Slept for 2½ hr
9/ 5/48	0230	3 mg Nu 2206	Relief
	0800	3 mg Nu 2206	Relief for about 2 hr
	1520	3 mg Nu 2206	Slept more than 2 hr
	2050	3 mg Nu 2206	Slept about 2½ hr
9/ 6/48	0030	3 mg Nu 2206	Relief
	0500	3 mg Nu 2206	No relief
	1545	3 mg Nu 2206	Slept more than 2 hr
	2330	3 mg Nu 2206	Asleep within ½ hr
9/ 7/48	0600	3 mg Nu 2206	Asleep within 15 min
9/ 8/48	0345	3 mg Nu 2206	

Control of pain was sought by 30 mg of morphine every four hours during the early stages of hospitalization. This dose was reduced as circumstances allowed, and on August 30, 100 mg Demerol were substituted for morphine. A tabulation of the drugs given this patient is presented in Table II.

## NU 2206 IN THE TREATMENT OF INTRACTABLE PAIN IN CANCER

Five patients with intractable pain due to cancer were observed for varying periods of time up to six months. The following case is presented in detail and is illustrative of some of the effects observed during a prolonged course of administration.

Patient G M, a 55 year old white woman was admitted to the University Hospitals on Oct 28 1947 with a diagnosis of carcinoma of the cecum. At operation a carcinoma of the ascending colon with regional lymph node metastasis was found and a right hemicolectomy was performed. She returned to the hospital on Jan 3 1949 with a large mass in the right upper quadrant of the abdomen which appeared to be liver involved by metastatic carcinoma. A posteroanterior radiogram of the chest revealed elevation and nodular outline of the diaphragm. The patient had been having very severe pain for two weeks and her physician had been unable to keep her free of pain by intermittent injections of morphine sulfate and Demerol. At the time of her admission she was emaciated and was apparently losing ground rather rapidly although she was still able to be out of bed for short periods of time. A right splanchnic block was done; this afforded temporary relief of the pain. This was repeated

TABLE III PATIENT G M

DATE	TIME	DRUG	RESULTS
1/8/49	1215	6 mg Nu 2206	Pain relieved in 45 min
	2015	6 mg Nu 2206	Pain relieved in 1 1/2 hr
1/9/49	1615	6 mg Nu 2206	Pain relieved in 45 min
1/10/49	1700	6 mg Nu 2206	Pain partially relieved in 30 min completely relieved in 60 min
			Before pulse 128 resp 28
			After pulse 124 resp 24
1/11/49	1100	6 mg Nu 2206	No record of result
	2030	6 mg Nu 2206	Sleeping soundly in 2 1/2 hr
			Before pulse 112 resp 24
			After pulse 96 resp 20
1/12/49	0655	6 mg Nu 2206	Sleeping soundly at 0730
1/13/49	1415	100 mg Demerol	1415 pain relieved Almost asleep
1/16/49	0930	10 mg Demerol	1000 sleeping at intervals
1/17/49	1000	6 mg Nu 2206	45 min stated she was not relieved Later in day stated she spent a comfortable afternoon
			Before pulse 120
			After pulse 112
1/21/49	1930	6 mg Nu 2206	2010 asleep
	1920	6 mg Nu 2206	2045 no pain
			Before pulse 124 resp 28
			After pulse 120 resp 20
	2330	6 mg Nu 2206	0200 asleep
1/22/49	0000	6 mg Nu 2206	2100 no pain 0200 asleep
1/23/49	1930	6 mg Nu 2206	2000 drowsy 0100 asleep
1/24/49	2000	6 mg Nu 2206	2100 asleep
1/25/49	1930	6 mg Nu 2206	2000 dozing
1/28/49	0000	6 mg Nu 2206	2100 asleep
1/29/49	19 0	6 mg Nu 2206	2000 asleep Slept all night
1/30/49	1930	6 mg Nu 2206	2100 asleep
1/31/49	0045	6 mg Nu 2206	0600 asleep
	2000	6 mg Nu 2206	Pulse 120 resp 30 Pulse thready
	2200	6 mg Nu 2206	Asleep Expired 2140 014,

once without satisfactory analgesia. Cordotomy and prefrontal lobotomy were considered but because of the patient's poor general condition and the short life expectancy, it was deemed advisable to try to obtain relief from pain by using analgesic agents rather than to subject the patient to a hazardous operative procedure. As may be seen from Table III, she received a total of twenty-three doses of 6 mg of Nu-2206 subcutaneously. This was given from January 8 to January 31 when she expired. The degree of relief accomplished by this means was considerable, and particularly gratifying was the long period of analgesia afforded. It was usually possible for her to sleep throughout the night without awakening with pain. This was in marked contradistinction to what had been observed with 10 mg doses of morphine and 100 mg doses of Demerol. In two instances during the period the patient was in the hospital, 150 mg of Demerol were given and the result appeared to be less enduring than with Nu-2206 in a dose of 6 milligrams.

In one urological patient who had an inoperable retroperitoneal tumor with continuous severe pain, Nu-2206 in repeated doses of 4.5 mg twice daily for a total of 113.5 mg in twenty-three doses was given throughout his stay in the hospital. He required no other analgesic drug.

In another patient, a 75-year-old white man (Patient E. T.) who had severe pelvic pain following recurrence and local metastasis of carcinoma of the rectum after abdominal perineal resection, Nu-2206 proved to be very effective in producing analgesia. A total of 111 doses, totaling 353 mg, was given over a period of sixteen weeks. Three milligrams proved sufficient when given at bedtime to produce comfort throughout the night so that the patient did not require other analgesic agents or barbiturates for rest. Occasionally during the daytime he took small doses of aspirin (0.6 Gm) and in a few instances 60 mg doses of codeine sulfate. During the first five weeks on Nu-2206, he received only eight doses of codeine. After he had received the drug for seven and one-half weeks, complete withdrawal was done for five days. The patient had no symptoms attributable to withdrawal. Examination five days after withdrawal showed no signs indicating withdrawal effect. Due to severe pain the patient was again placed on the drug and the dose was increased to 5 mg shortly before death. After fourteen weeks he developed symptoms and then signs indicating subtentorial brain metastasis. Three weeks later he expired. No Nu-2206 was given during the last six days of life because of coma.

A summary of our experience with Nu-2206 in the treatment of pain in patients with cancer is presented in Table IV.

Naturally the question of habituation and withdrawal effects is also raised. In addition to the instance already cited (Patient E. T.) in which withdrawal of the Nu-2206 was done after many weeks, ten other patients were studied in this regard. These all received the drug for much shorter periods. None of them demonstrated withdrawal effects. This experience is summarized in Table V.



TABLE IV. NUMBER IN TREATMENT OF PAIN OF CANCER

PATIENT	SEX	AGE	INDIVIDUAL DOSE (mg)	NUMBER OF DAYS ADMINISTERED	TOTAL DOSE (mg)	EFFECT	SIDE EFFECTS	DIAGNOSIS OF TUMOR (exploratory thoracotomy)
C.H.	M	41	10 mg	4	40 mg	Maximal	None	Carcinoma of lung
G.M.	F	51	10 mg	10	100 mg	(Good) maximal	None	Mitotic carcinoma to liver
I.A.	F	55	10 mg	3	30 mg	Maximal	None	Carcinoma of rectum metastasis in lung
C.W.	F	51	10 mg	7	70 mg	Slight moderate	None	Mesothelioma of pleura
B.S.	M	58	40 mg	10	400 mg	Maximal	None	Inoperable inoperable tumor
D.	F	50	40 mg	6	240 mg	(Good) maximal	Slight hiccups	Carcinoma of cervix
K.	F	40	40 mg	8	320 mg	Slight moderate	None	Carcinoma of cervix
T.	F	50	40 mg	4	160 mg	Moderate	None	Carcinoma of vulva
C.	F	61	10 mg	4	40 mg	Maximal	None	Carcinoma of cervix
F.F.	M	50	10 mg	30	300 mg	Maximal	No undesirable ones	Recurrent and metastatic carcinoma of rectum in pelvis

TABLE V ABSENCE OF WITHDRAWAL EFFECTS OF NU 2206

PATIENT	SEX	AGE	INDIVIDUAL DOSAGE	NUMBER OF DAYS BEFORE DRUG WITHDRAWN	TOTAL DOSAGE	EFFICACY	SIDE EFFECTS	DIAGNOSIS OR PROCEDURE	WITH DRAWAL SYMPTOMS
E H	F	34	5 mg	6	130 mg	Maximal	Nausea and vomiting, two times a day	Metastatic epidermoid carcinoma of cervix to 2nd, 3rd, 4th ribs, rt	None
M M	F	82	5 mg	5	60 mg	Good	Pulse irreg- ular	The double nerve section	None
E S	M	63	6 mg	10	84 mg	Maximal	None	Bronehogene carcinoma (Rt pneumonec-tomy)	None
H N	M	53	6 mg	15	84 mg	Maximal	None	Metastatic carcinoma in pelvis Previous abdominal perineal resection	None
C B	M	71	6 mg	10	78 mg	Maximal	None	Inoperable carcinoma of lower lobe of left lung	None
E H	M	57	5 mg	2	12 mg	Maximal	None	Splenectomy for Banti's syn- drome	None
E B	M	48	5 mg	7	70 mg	Maximal	None	Phantom limb pain	None
G T	M	52	50 to 100 mg	12	590 mg	Maximal	None	Malignant lymphoma with metas- tasis to spinal cord	None
R B	M	42	3 mg	1	30 mg	Maximal	None	Retropitoneal undifferentiated malignant neoplasm	None
M B	F	62	5 mg	3	40 mg	Maximal	None	Multiple sclerosis Cordotomy done	None

## SUMMARY

- 1 Nu 2206 has desirable analgesic properties when administered subcutaneously in doses of 30 to 60 milligrams
- 2 For most adults 15 mg Nu 2206 is an ineffective dose
- 3 The duration of analgesia is more prolonged than with 10 to 15 mg morphine sulfate
- 4 Untoward reactions to Nu 2206 are not severe or frequent
- 5 The prolonged effect of Nu 2206 is beneficial in those patients who require protracted relief and in whom frequent injections with shorter acting analgesics are undesirable
- 6 No withdrawal symptoms have been observed in this study

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# THE EFFECT OF SPLEEN PROTECTION ON MORTALITY FOLLOWING X-IRRADIATION

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LEAD protection of the surgically mobilized spleen of CF-1\* mice during the delivery of 600 r total body x-radiation obviates the development of anemia and significantly lessens the severity and duration of the leucopenia and thrombocytopenia that regularly follow total body exposure at this level<sup>1</sup>. On the basis of previous work,<sup>2</sup> a single total body exposure to 550 r x-radiation produces death of one half of the mice of this strain in twenty eight days (LD<sub>50</sub>/28 days) †. Survival data on groups of mice exposed to single dosages of total body radiation with or without lead protection of the surgically mobilized spleen are presented in this paper.

## MATERIAL AND METHODS

The mice used in this study were all females and were 10 to 12 weeks of age when the experiment was initiated. The mice were kept in the animal farm in a constant temperature room (74° F) for four to six weeks before use and were maintained on a diet consisting of DeWood<sup>‡</sup> and water ad libitum before and after the experimental procedures described below.

**Dosimetry**—The x-rays administered in these experiments were generated in a 250 kv machine operating at 15 milliamperes. A 0.25 mm copper filter was used. The half value

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\*CF-1 raised by Calworth Farms. Homozygous for the recessive genes aa bb cc.

†Hereafter referred to as LD<sub>50</sub>.

‡Manufactured by the DeWood Mill DeWood Md. Consists of skim milk ground wheat yeast corn oil salt iron citrate shark liver oil Delsterol.

TABLE I SURVIVAL OF MICE FOLLOWING SINGLE TOTAL BODY

GROUP	DOSAGE (r)	SPLEEN SHIELDED	TOTAL NUMBER OF MICE	NUMBER OF SURVIVORS*	PERCENT SURVIVAL
IV	600	Yes	63	54	86
III	600	No	75	30	40
IV	700	Yes	24	26	108
III	700	No	11	0	0
IV	900	Yes	60	41	68
III	900	No	44	3	6.8
IV	975	Yes	23	10	43.4
III	975	No	12	0	0
IV	1,050	Yes	23	7	30.4
III	1,050	No	11	0	0
IV	1,200	Yes	19	0	0
II	0	Operation only	64	64	100
I	0	No operation	54	52	96

\*On Oct. 3, 1949, five or more months after these exposures all mice which had lead protection of the spleen during irradiation still survive and appear grossly normal.



layer in copper of the filtered beam using 240 kv was 1.0 millimeter. The exposures were measured with a Victoreen condenser meter equipped with a 250 r chamber. Measurements were made in air at the position occupied by the center of the animal's body. The dose rate averaged 58.9 r per minute at 55 centimeters.

Groups of mice were exposed to single doses of 600, 700, 900, 975, 1,050, and 1,200 r total body  $\gamma$  radiation with or without lead protection of the surgically mobilized spleens.

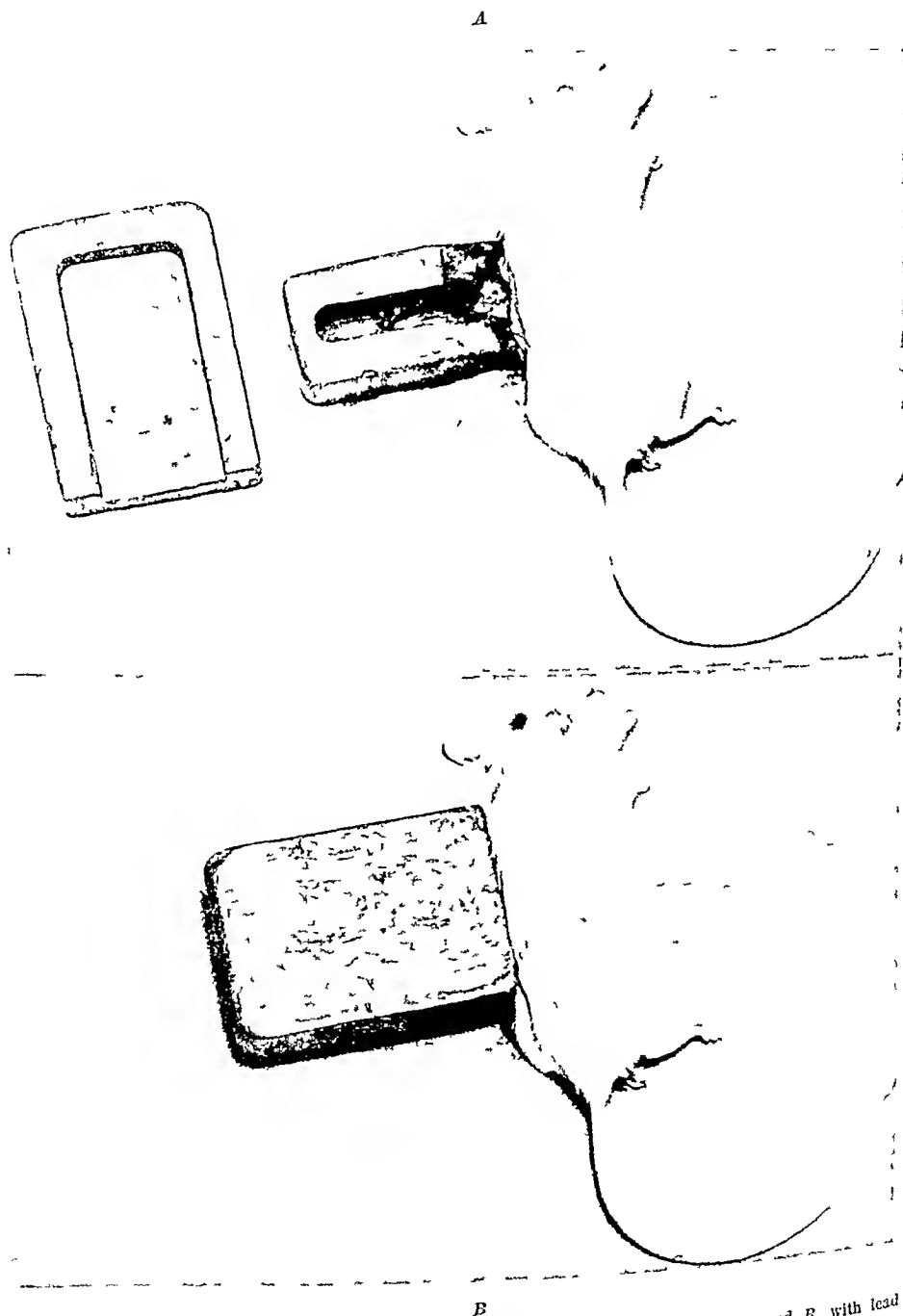


Fig 2—A, Photograph of a mouse with mobilized spleen within lead box and B, with lead cover in place

## EXPERIMENTAL PROCEDURE

As is indicated in Table I four groups of mice were studied. Mice in Group I were untreated controls. The mice in Groups II, III and IV were anaesthetized with Nembutal (0.072 mg per gram mouse in a 1:10 dilution) given intraperitoneally; the abdomen was shaved, an incision was made in the left upper quadrant and the spleen was brought out through the incision. The main pedicle was left intact but to facilitate mobilization a small

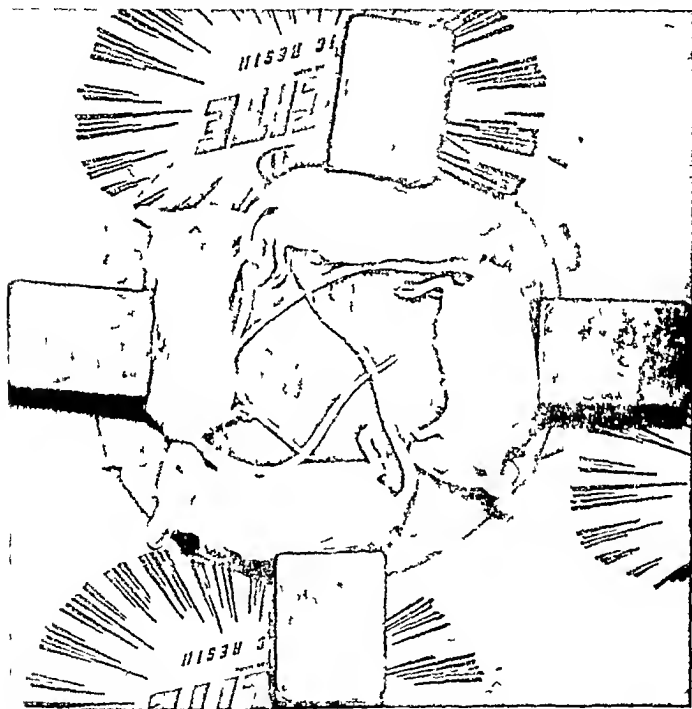


Fig. 3—Photograph of mice showing surgically mobilized spleens within lead boxes. The mice were irradiated in this arrangement.

vessel at the distal end of the spleen was cut but not tied. Severing this vessel regularly produced an infarct in the tip of the spleen. The infarct varied in size involving up to one-fourth of the distal tip. No significant bleeding was encountered. During irradiation which required from nine to twenty-two minutes depending on the dose given the spleens of Group III mice were placed in paraffin boxes that offered no appreciable shielding (Fig. 1); the spleens of Group IV mice were placed in lead boxes with walls of one-fourth inch thickness (Fig. 2 A and B and Fig. 3) that afforded essentially complete shielding of the spleen from irradiation. The spleens of Group II were kept in lead or paraffin boxes during the x-irradiation.

tion of Groups III and IV. The spleens of all groups thus mobilized were kept moist with physiologic saline during the period in which they were outside the body cavity. After completion of these procedures, the spleens of all groups were returned to the abdominal cavity and the incision was sutured with silk. Complete recovery from the anesthetic required from two to three hours. The mice were returned to the animal farm, and deaths were recorded in twenty four hour periods through twenty eight days.

#### RESULTS

As indicated in Table I, the percentages of survival of animals in Groups I and II (untreated controls and operated controls) were 96 and 100 per cent respectively. With a dosage of 600 r, 40 per cent of the animals in Group III survived the twenty-eight day period. Accordingly, the  $LD_{50}$  for animals whose spleens were mobilized but not protected was somewhat less than 600 r and probably close to the  $LD_{50}$  of intact animals (550 r). On the other hand, in mice of Group IV which were provided with lead protection of the spleen during irradiation of the balance of the body, a dosage of 975 r was required to reduce survival to about 50 per cent. In other words, these data show that the  $LD_{50}$  for total body  $\alpha$ -irradiation exclusive of the lead-protected spleen is nearly twice as great as the  $LD_{50}$  for total body  $\alpha$ -irradiation inclusive of the spleen.

#### DISCUSSION

The precise mechanism whereby lead protection of the spleen so significantly increases survival following irradiation is yet to be determined. The capacity of the spleens of these mice to compensate so quickly for the destruction of hematopoietic tissue elsewhere in the body may be the significant factor, but other possible direct or indirect functions of the spleen may be involved. For example, data from a preliminary experiment<sup>3</sup> indicate that rabbits retain the capacity to produce antibodies to intravenously administered foreign red cells (1 cc of a 2 per cent suspension of sheep red cells) in a normal manner if either the spleen or appendix is lead protected during the delivery of 800 r  $\alpha$ -irradiation to the balance of the body, total body  $\alpha$ -irradiation with this dosage but without lead protection of the spleen or appendix more or less completely inhibits the development of demonstrable antibodies to this antigen. The antigen was given two days after irradiation and observations on the development of hemolysis titer followed at intervals of seven days through twenty eight days.

#### SUMMARY

Female CF-1 mice of 10 to 12 weeks of age were divided into four groups. Group I mice were untreated controls. Mice in Groups II, III, and IV were anesthetized with Nembutal, an incision was made in the left upper quadrant of the abdomen, and the spleen was mobilized. Mice in subgroups of Groups III and IV were given various single dosages of  $\alpha$ -irradiation. During irradiation the mobilized spleens of Group IV mice were protected from radiation by lead shielding, whereas no shielding was provided for the spleens of mice in Group III. Group II mice served as "operated" controls. The dosages administered were 600, 700, 900, 975, 1,050, and 1,250 r. Irradiation time ranged between



nine and twenty two minutes. After irradiation the spleen was returned to the abdominal cavity and the incision was sutured. The survival of the mice in these various groups was observed over a twenty eight day period. Without lead protection of the spleen the  $LD_{50}$  for irradiated mice in the twenty eight day period of observation was less than 600 r, whereas the  $LD_{50}$  for mice with lead protection of the spleen approximated 975 r.

#### CONCLUSIONS

The  $LD_{50}$  for mice exposed to total body  $\gamma$  radiation exclusive of the surgically mobilized lead protected spleen is nearly twice as great as the  $LD_{50}$  for mice exposed to total body  $\gamma$  radiation inclusive of the spleen.

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# OBSERVATIONS ON MECHANISMS OF EDEMA FORMATION IN THE LUNGS

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## INTRODUCTION AND REVIEW OF LITERATURE

**K**NOWLEDGE of the pathologic physiology of pulmonary edema has been considered fundamental to the understanding and management of clinical heart failure, and the condition has received extensive study. From a great body of experimental and clinical observation, two theoretical conceptions of the genesis of pulmonary edema from heart failure have emerged.<sup>1, 2</sup> First, many observers have stated that pulmonary edema depends on failure of the left ventricle (the right remaining essentially competent), leading to congestion and edema of the lungs. The second concept has held that pulmonary edema occurs when the pulmonary vascular bed becomes widely dilated from noxious or aberrant reflexes permitting inundation of the lung tissue. This concept has further held that pulmonary edema is not caused primarily by failure of the left ventricle. The importance of the problem of pulmonary edema has seemed to warrant a re-exploration of the condition from the experimental standpoint.

In 1878 Welch<sup>3</sup> stated, in his now famous paper, that severe strain imposed upon the left ventricle by gradual occlusion of the aorta resulted in lung congestion and edema. His experiments indicated that ligation of the aorta distal to the large vessels arising from the arch did not restrict left ventricular output sufficiently to strain the chamber. However, if the vessels to the head and upper extremities were tied off one by one after occlusion of the aorta distal to these vessels, the vascular bed was finally reduced so as to overwhelm the left ventricle, marked pulmonary congestion and transudation followed. Subsequently, a number of German workers were able to offer general confirmation of Welch's observations. Sahli<sup>4</sup> found that obstruction of pulmonary venous drainage by constriction of the left auricle was attended by an outpouring of fluid into the lungs. Sahli was unable to reproduce Welch's results by ligation of the aorta, but it is not clear from his paper where aortic constriction was applied. Other observers<sup>5, 6, 7, 8, 9, 10, 11, 12, 13, 14</sup> were able to show that experimental maneuvers capable of overloading the pulmonary venous circuit could precipitate pulmonary edema. Modrakowski<sup>8</sup> demonstrated that isolated lungs could withstand high arterial perfusion pressures without apparent harm, as long as venous outflow was unimpaired. On the other hand, when the lobes were congested by the prevention of venous outflow, only a moderate elevation of perfusion pressure was necessary to provoke edema.

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Similar experiments were carried out on denervated heart lung preparations<sup>9 10 11</sup>. The data from these experiments indicated that as left ventricular output was diminished (e.g., from cardiac damage by asphyxia "toxic factors," or acute strain), venous engorgement of the lungs occurred, ultimately causing severe edema. The work of Barry<sup>1</sup> was of particular interest. He observed in heart lung preparations that if the viscosity of the blood was decreased by the addition of saline edema of the lungs could be induced. This effect was apparent when the specific gravity of the blood was reduced from 1.053 (normal) to 1.045 or 1.050. However Barry also observed in these preparations that excessive venous inflow, together with high peripheral arterial resistance overloaded the left ventricle so that congestion and alveolar edema ensued. Barry's observations were of significance in their demonstration that the Starling principles<sup>13</sup> of vascular pressures and tissue fluid balance may apply to the lung as well as to structures supplied by the peripheral vascular system.

On the basis of such experiments the concept of pulmonary engorgement and transudation from predominant left ventricular failure has been widely accepted, and much of the rationale of the management of clinical heart failure has rested upon this premise.

Many observers, however, have accepted the alternative concept that pulmonary edema may result from intense reflex vasodilatation of the vascular bed of the lungs or from increased capillary permeability. They have argued that failure of the heart may not be primarily concerned in initiating pulmonary edema. Lambert and Giemels<sup>15</sup> insisted that the formation of edema in heart lung preparations was accompanied by only a slight rise of pulmonary venous and arterial tensions. These pressures became significantly elevated after edema was established. They thought the capillary leakage was caused by the "toxicity" of the shed blood used in the preparations. A number of others<sup>12 16 17 18</sup> have shared the view that toxic factors occurring in the blood may injure the pulmonary capillaries and facilitate transudation.

Cataldi<sup>19</sup> used a technique similar to that of Coelho and Rocheta<sup>14</sup> and he noted the precipitation of pulmonary edema when portions of the right ventricle were destroyed by solutions of silver nitrate or absolute alcohol introduced into the ventricular wall. He contended that failure of the left ventricle was not a necessary prerequisite to the onset of pulmonary edema, for damage to the right ventricle might be equally deleterious to the lungs.

The contention that pulmonary vascular reflexes may lead to lung edema formation has rested largely on observations entailing marked disturbances of the sympathetic nervous system<sup>19 20</sup>. Luisada<sup>19</sup> produced paroxysms of edema in rabbits by the use of adrenalin. The paroxysms were often prevented by the use of sedatives (i.e. morphine or the barbiturates), or by transection of the spinal cord at the cervical level. He attributed these effects of adrenalin to stimulation of the central nervous system including centers controlling pulmonary vascular reflexes and capillary permeability. In contrast evidence has been evolved to show that adrenalin may seriously affect the heart<sup>21</sup> and provoke myocardial failure. Luisada and Sainoff<sup>22</sup> declared that experimental

procedures producing cerebral anemia may alter the distribution of blood within the lungs by vasomotor mechanisms, so that alveolar transudation occurs. A similar explanation was suggested by Janisch and co-workers<sup>23</sup> for the edema resulting from the intracisternal injection of veratrine in rabbits. Farber<sup>24</sup> reported that edema of the lungs frequently occurred in guinea pigs following bilateral cervical vagotomy causing the loss of vasomotor control of the lungs. However, Sussman and co-workers<sup>25</sup> could find no such effect of vagotomy in guinea pigs when respiration was optimally maintained.

On the basis of clinical observation, a number of workers have suggested that pulmonary edema may be touched off by failure of autonomic control of the pulmonary vascular bed. Salmon<sup>26</sup> implicated stimulation of the carotid sinus as a means of inducing transudation. Intracranial hemorrhage<sup>31</sup> and injuries of the spinal cord<sup>32</sup> and of the medulla oblongata<sup>33</sup> have likewise been reported to cause intense pulmonary edema.

In most of the clinical and experimental observations of "neurogenic" pulmonary edema, measurements of cardiac performance were not reported. If the function of the heart were seriously curtailed through aberrant autonomic stimulation from various causes, one might expect lung congestion and its consequences from a critical decrease of left ventricular output. In fact, Campbell, Haddy, and Visscher<sup>34</sup> have shown recently that elevation of intracranial tension may be accompanied by marked bradycardia, diminution of cardiac output, and elevation of pulmonary venous and arterial pressures, with the occurrence of pulmonary edema. These responses were modified by severance of the vagus nerves.

The conflicting views of the genesis of pulmonary edema, as indicated in this review, have not been reconciled. Luisada has stated that "the generally unsatisfactory basis for the backward failure theory of pulmonary edema strongly favors its abandonment."<sup>35</sup> On the other hand, there is a paucity of evidence to support the "neurogenic theory" as the sole cause of edema of the lungs. Furthermore, other evidence has indicated that the latter concept must be viewed with skepticism until reflex effects upon the heart and its function have been investigated more thoroughly.

It seems clear however that any attempt to explore the cause of pulmonary edema requires an understanding of the means by which abnormal fluid exchange may occur in the lungs. Although the concise precepts of fluid balance described by Starling<sup>13</sup> are widely accepted as applying to the lung as well as to other tissues, there has been little controlled observation of the Starling principles on the lungs. Experiments were therefore designed to test the effects of increased capillary hydrostatic pressure and of diminished osmotic tension (the Starling principles) on pulmonary circulatory function. Experiments dealing with this aspect of the problem will be reported in this paper.

#### METHOD

Experiments were performed on dogs under Nembutal anesthesia. The right thoracic duct was isolated, and the rate of lymph flow was measured by the method previously described by us.<sup>35</sup> Under positive pressure respiration, the thorax was opened and the

pericardium incised. The animal was then heparinized in the usual manner after careful hemostasis had been secured. Systemic and pulmonary arterial pressures were recorded from cannulae placed in the left abdominal artery and right upper pulmonary lobar artery, these were connected to simple mercury manometers. A water manometer was attached to a cannula tied into the left atrial appendage. Ordinary heart lung preparations were then set up.

In order to study the effects of decreased osmotic pressure the protein content of the blood was reduced by partial replacement of the plasma with Locke's solution (six experiments). Dilution was accomplished by centrifuging a quantity of blood approximately equal to that in the circuit; the plasma was removed to the level of the packed cells by suction, and the original plasma volume was then restored by the addition of warmed Locke's mixture. At any given time the normal blood in the venous reservoir could be drained away and replaced by the red cell suspension. The rates of lymph flow, the various pressures, and the appearance of the lungs were repeatedly observed. Sections of the lungs for histologic examination were taken during and at the termination of the experiments. Plasma protein levels were determined by the methods of Campbell and Hanna<sup>32</sup> and of Howe.<sup>33</sup>

The effect of increased pressure in the pulmonary capillaries on edema formation was studied in six preparations. Pulmonary engorgement was produced by overloading the left ventricle. Elevation of venous inflow (and therefore cardiac output) or increase of the peripheral resistance singly or together produced left ventricular dilatation and left atrial engorgement. The effects of this treatment on pulmonary arterial and venous pressures and on blood volume were noted. Lymph flow was frequently determined and sections for microscopic study of the lungs were taken.

All specimens of lung tissue were fixed in formal saline solution (Trowell<sup>3</sup>) so as to facilitate preservation of edema fluid and were stored in 80 per cent alcohol.

## RESULTS

*Effects of Diminution of Plasma Proteins on the Formation of Pulmonary Edema*—During the control periods the heart lung preparations functioned under loads that appeared to place no strain upon the myocardium. Usually the peripheral resistance was adjusted to produce a mean aortic pressure of 90 to 110 mm Hg; the cardiac inflow was about 400 cc per minute. Under these conditions mean pulmonary arterial pressure varied from 10 to 20 mm Hg, and left intra atrial tension ranged from 3 to 6 cm of water. Thereafter the suspension of cells in Locke's fluid was placed in the venous reservoir after removal of as much of the normal blood from the circuit as possible without introduction of air into the venous tubing. Usually 200 to 300 cc of the cell suspension were used. In most experiments it was estimated that approximately one half of the circulating blood volume was replaced when these quantities of cell suspension were used.

The effects of the addition of diluted blood were first manifested in the flow of lymph from the lungs. The flow increased notably within one to ten minutes and thereafter was progressively accelerated. The level of blood in the venous reservoir decreased indicating a loss of circulating blood volume. However, the size of the heart was not increased and pressures in the pulmonary arteries and left atrium were not materially altered. In four experiments pulmonary arterial pressure was not affected at all; in one instance a decrease of 4 mm Hg tension was noted while in two examples there were transient elevations of the pulmonary blood pressure of 5 mm Hg. Pulmonary venous pressures were unaltered in two instances and rose from 2 to 6 cm of water in four

preparations Therefore, the continued normal size of the heart and the general level of performance indicated that cardiac function was not impaired (Fig 1 and Table I)

After the cell-saline suspension was circulating in the preparations, and during the period of fluid loss into the lungs, there was a gradual return of plasma protein concentration to normal values In some instances the plasma

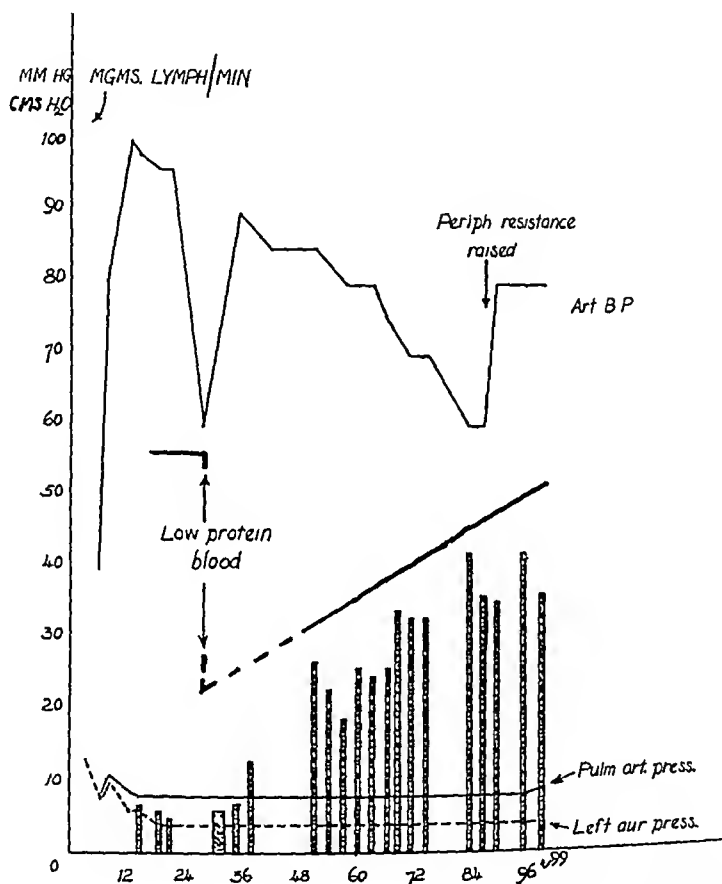


Fig 1—The effect of hypoproteinemia upon fluid exchange in the lungs. At the time indicated low protein blood was placed in the heart lung system. Protein concentration of the blood is indicated by curve. Columns represent lymph flow. Abscissa is time in minutes. See Table I Experiment 48.

proteins continued to rise to concentrations greater than those in the normal, undiluted blood (See Table I). However, with the rise of plasma protein concentrations to control (or above control) values, a corresponding diminution of lymph flow and reduction of pulmonary edema was *not* observed. This phenomenon is well illustrated in Fig 1. Note that the curve indicating plasma protein concentration of the cell suspension showed a progressive increase, but that lymph flow also showed a continuous rise. The persistence of accelerated lymph flow after the return of plasma proteins to normal levels probably represented the drainage of the previously accumulated fluid from the lungs. However, the mechanism by which the plasma proteins subsequently rose to levels

TABLE I EFFECT OF DIMINUTION OF PLASMA PROTEINS

EXPERIMENT	CHANGE IN		LYMPH FLOW		PLASMA PROTEIN			PUL MONARY EDEMA
	PUL MONARY VITAL PRESSURE (MM /HG)	LEFT AURICULAR PRESSURE (CM /H O)	CONTROL (NO / MIN)	AFTER DILUTION (MG / MIN)	CONTROL (GM %)	DILUTED (GM %)	FINAL (GM %)	
47	+2 to +2	+2 to +2	40	180		31	70	1 resent
48	0	0	5	42	5.5	31	58	Present
49	+5 to 0	+1	6	16	5.2	23		Present
51	0	+1	16	18	6.5	21	85	
53	0	+6			5.5		98	Pre ent
54	0	0	98 11	150 11	7.4		82	Present

above control values is not clear. Possibly the interstitial fluid produced by the initial hypoproteinemia obstructed aeration of the lung tissue causing anoxic damage to the pulmonary capillaries and abetting transudation of protein poor fluid. Another explanation may be that plasma protein lost into the extravascular space during initial transudation increased tissue fluid osmotic tension to such a degree that transudation went on despite the increase of blood protein level, producing greater hemoconcentration.

*Influence of Cardiac Insufficiency and Pulmonary Congestion on the Formation of Edema of the Lungs*—These observations were carried out on six heart lung preparations. For control purposes the venous inflow and peripheral resistance were adjusted so as to impose no undue strain upon the hearts as in the preceding experiments. In four experiments the cardiac load was augmented by increasing the aortic pressure from 90 to 110 mm Hg to 160 to 180 mm Hg. In two instances, overburdening of the heart was accomplished by sharp elevations of venous inflow to 600 to 700 cc per minute (control 200 to 300 cc per minute). With both means of taxation of the heart left ventricular dilatation became apparent. The pulmonary venous and arterial pressures then rose and congestion promptly ensued. Congestion of the lungs was manifested by turgescence, followed quickly by dusky of the tissue. Myriads of fine crepitant rales could be discerned by direct auscultation. The fluid volume in the venous reservoir diminished rapidly, and the passage of lymph from the right thoracic duct progressively increased beginning three to twelve minutes after the onset of congestion. As the process continued the pulmonary lymph became pinkish in hue and finally dark red. Microscopic examination of the red lymphatic fluid showed that it contained up to 600 000 red blood cells per cubic millimeter. Sections of the lung tissue taken at the time of accelerated lymph flow exhibited intense congestion and edema. In some instances pulmonary transudation was so mired that foam extended into the main bronchi and trachea. In all of these experiments pulmonary engorgement and edema was permitted to become extreme, no attempt was made to reverse the process by reducing cardiac load.

The difference in appearance between these lungs and those of the hypoproteinemic experiments was striking. When the hypoproteinemic lungs became edematous, they were pale and flaccid, the acceleration of lymph flow was not so rapid, and the general tempo of the reaction was rather gradual. In contrast the congested lungs became red and turgid, the acceleration of lymph flow was rapid, and the lymph became hemorrhagic. Edema fluid was frequently profuse.

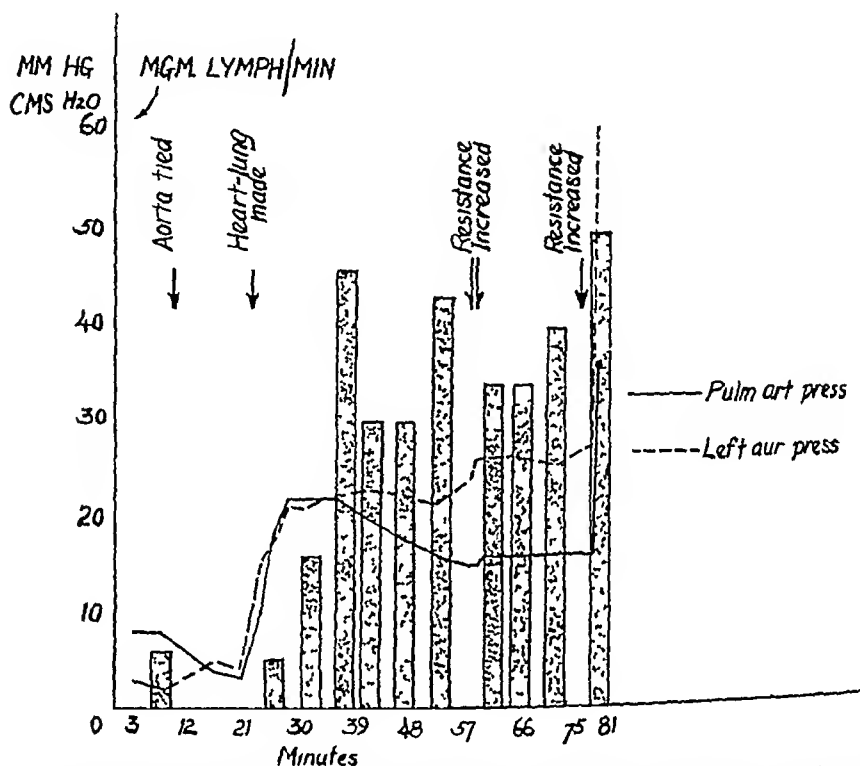


Fig. 2—The effects of pulmonary congestion upon fluid exchange in the lungs. When the heart-lung was established in this experiment, peripheral resistance was excessive and pulmonary congestion immediately appeared as indicated by venous and arterial pressure curves. Congestion was further increased at arrows. Columns represent lymph flow.

#### DISCUSSION

These experimental data suggest that the well-known Starling principles of vascular and tissue fluid balance are operative in the lung structures. The mechanism of pulmonary edema induced by low plasma protein—low blood osmotic tension—is clear and requires no particular comment. However, the production of pulmonary edema from congestion is more important from the standpoint of cardiac insufficiency.

The experiments show that in the heart-lung preparation, left ventricular load may be greatly augmented by elevation of aortic pressure or by increasing the stroke and minute output or by both. Thus the left ventricle can be overloaded so that venous inflow (and right ventricular output) may exceed the greatest possible left ventricular output. With failure of the left ventricle to accept all of the blood flowing to it through the lungs, pulmonary venous



outflow is impaired and blood is impounded in the lungs by the continuing competence of the right ventricle. Examination of Fig 2 and Table II shows that these events cause pulmonary venous and arterial pressures to rise to high levels. Since the normal pulmonary capillary pressure is estimated at mean values of 10 to 15 mm Hg in open chest preparations<sup>33</sup> and the normal blood osmotic pressure at 20 mm Hg (judged by normal plasma protein levels in these and other<sup>39</sup> experiments) it is apparent that capillary pressure may exceed that of the osmotic tension when the lungs are congested. Rapid filtration of fluid from the capillaries then occurs.

TABLE II EFFECTS OF PULMONARY CONGESTION

EXPERIMENT	RISE IN		LYMPH FLOW		PULMONARY EDEMA	CARDIAC OUTPUT (CC/MIN)
	PULMONARY ARTERIAL PRESSURE (MM/HG)	LEFT AURICULAR PRESSURE (CM/H <sub>2</sub> O)	CONTROL (MG/MIN)	AFTER CONGESTION (MG/MIN)		
35		59	65	145	Present	
38	56	57	6	63	Present	
39	48	74	40	180	Present	
40	50	84			Present	
41	34	50			Present	500 to 650
43	5	14			Present	160 to 310

A contrasting explanation for experimental pulmonary edema was offered by Luisada and Sarnoff<sup>22</sup>. They reported experiments in which saline infusions, given in large quantity, produced pulmonary edema. They noted that when the solutions were injected cephalad into the carotid arteries under high pressure edema of the lungs frequently occurred. On the other hand when comparable amounts of fluid were injected into the systemic veins under lower pressure, edema was somewhat less frequent. They suggested that forcible irrigation of the cerebral circulation by salt solutions resulted in cerebral anemia and provoked pulmonary transudation by reflexogenic influences on the vascular bed of the lungs. Their results were compatible with sudden and serious cardiac overload. They paid scant attention to the possibility of altered circulatory dynamics, and the influence of massive saline infusions on the plasma proteins was not considered.

Other workers<sup>40-41</sup> have also reported that massive increases in the circulating fluid volume may introduce a load against which the heart cannot cope, with consequent pulmonary congestion and edema. This factor was particularly striking in the studies of Gibbon and Gibbon.<sup>7</sup> In cats they removed the right middle and both lower lung lobes so as to raise the pulmonary hydrostatic pressure by restriction of the circulatory capacity of the lungs. They found that intravenous saline infusions were especially likely to provoke pulmonary edema and rapid death in these preparations. Although these workers attributed the edema mainly to increased capillary permeability rather than to any dynamic effects, their results suggest that edema occurred from elevation of the pulmonary hydrostatic pressure and possibly from diminution of the plasma proteins.

The correlation of increased lymph flow and the onset and progression of pulmonary edema was strikingly evident in these experiments. We noted that in every instance where a significant elevation of lymph flow occurred, gross or microscopic pulmonary edema could be found.

#### SUMMARY

Experiments are presented which show that pulmonary edema may be induced in the heart-lung preparation by (1) the lowering of the plasma proteins from replacement of blood plasma with Locke's solution and (2) by elevation of pulmonary vascular (hydrostatic) pressures following imposition of left ventricular overload. It was demonstrated that abnormal fluid exchange in the lung tissue may result from disturbances of hydrostatic and osmotic pressure relationships as described by Starling.<sup>13</sup> Although the results of these experiments, involving the heart-lung preparation, do not necessarily explain the cause of acute pulmonary edema in intact subjects, they do demonstrate that Starling's principles apply to the pulmonary as well as to the systemic circulation. The Starling principles, therefore, must be considered in all future studies relating to the pathogenesis of pulmonary edema.

The onset and progression of pulmonary edema were always attended by an increase in the flow of lymph from the right thoracic duct. The measurement of an increased pulmonary lymph flow has been found to be a reliable indicator of the presence of pulmonary edema.

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## LABORATORY METHODS

### POLYVINYL ALCOHOL-FIXATIVE AS A PRESERVATIVE AND ADHESIVE FOR PROTOZOA IN DYSENTERIC STOOLS AND OTHER LIQUID MATERIALS

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TROPHOZOITES of intestinal amoebae deteriorate rapidly, and consequently it is frequently difficult for a physician to get laboratory confirmation of suspected cases of amoebic dysentery or amoebiasis. Submitting the stool to a distant laboratory for diagnosis has been unsatisfactory since the etiological agent, even if originally present, is usually unrecognizable by the time the specimen is examined. A negative report under these circumstances is, of course, meaningless.

In an attempt to solve this problem, Goldman<sup>1,2</sup> devised a technique for preserving fecal smears with a film of polyvinyl alcohol (PVA)-fixative. Smears so prepared could be shipped for long distances or stored in a dried condition for months without undergoing any deterioration. In the laboratory, the PVA films were removed and the smears were stained by standard hematoxylin procedures.

During 1948, this method was used by this laboratory in cooperation with local health officials and physicians in Georgia, Alabama, Mississippi, Kansas, North Dakota, Minnesota, and California. Out of thirty specimens submitted during several months, a diagnosis of *Endamoeba histolytica* was made in thirteen instances. The rest showed either no organisms or trophozoites and cysts of various other intestinal protozoa (*Endamoeba coli*, eleven, *Endolimax nana*, fifteen, *Dientamoeba fragilis*, one, and *Giardia lamblia*, one).

The purpose of the present paper is to report simplified and improved methods for preparing specimens and for handling them in the laboratory, and to point out possible applications of the technique to the study of organisms other than intestinal protozoa.

#### MATERIALS AND METHODS

*Polyvinyl Alcohol*—Polyvinyl alcohol is a synthetic water soluble polymer of vinyl alcohol that is available in the form of a white, odorless powder or granular material. Its aqueous solutions are stable over long periods of time. Viscosity of the solutions can be regulated

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by concentration of PVA and by the degree of polymerization of the alcohol\*. Films formed by drying aqueous solutions are tough thin and transparent. They generally adhere firmly to clean glass surfaces and are resistant to alcohol, ether, acetone, xylol, oils, and even short exposures to water.

PVA has been used as an embedding medium for tissues<sup>3</sup> as a mounting medium for insects and fungi<sup>4, 5</sup> and as a means of reducing motility of paramecia and other small organisms.<sup>7</sup>

**PVA Fixative**—The fixative was prepared as described by Goldman.<sup>2</sup> Five grams of powdered PVA were added to a mixture at room temperature containing 15 ml of glycerol, 5 ml of glacial acetic acid and 93.5 ml of Schaudinn's solution (2 parts of saturated aqueous mercuric chloride to 1 part of 90 per cent ethyl alcohol). Heating to approximately 75° C while stirring facilitated the preparation of a water-clear nonlumpy solution. This was used as soon as it had cooled to about 30° C or less and it remained satisfactory for several months.

#### *Preservation of Specimens With PVA Fixative*—

**A. On Microscope Slides** With the aid of an applicator stick, a drop of specimen was mixed on a microscope slide with 3 drops of fixative and smeared over approximately one third of the glass surface. To insure thorough drying smears were usually placed in an incubator at 37° C overnight.

**B. In Vials** A quantity of specimen was thoroughly mixed in a vial containing three or more parts of fixative. Smears for staining were prepared immediately or months later by spreading a drop or two of the mixture on a microscope slide and allowing it to dry thoroughly. Care was taken not to have the smears too thick.

**Staining of Organisms in PVA Films**—In the original PVA fixative technique, fecal smears were covered but not mixed with fixative. In the laboratory the films were removed before staining the smears. The present methods of preservation were made practical by the observation that PVA films are permeable to all commonly employed staining reagents. This makes it possible to stain dried PVA smears in the same manner as smears fixed by conventional methods with Schaudinn's fixative.

Inasmuch as critically stained organisms were desired, the long Heidenhain iron hematoxylin procedure usually was employed. Diagnostically satisfactory preparations were obtained by more rapid staining procedures such as the ones proposed by Tompkins and Miller<sup>8</sup> and Goldman.<sup>9</sup> Delafield's hematoxylin was used in some instances as in staining *Paramecium*. In all of the staining procedures dried films were first placed in 70 per cent alcohol containing iodine for ten minutes or longer in order to remove mercuric chloride crystals.

Ordinarily if the PVA film on a slide is thoroughly dried before being stained it adheres perfectly. In rare instances, for reasons not entirely understood, films may wrinkle around the edge or in the middle and show a tendency to slip off the slide during prolonged exposure to aqueous solutions. This tendency may be minimized by preparing relatively thin, rectangular smears. To prevent the loss of a wrinkling film the slide may be placed in the incubator in a horizontal position and allowed to dry before continuation of the staining process.

In order to test the effect of drying organisms in PVA films, such films were thoroughly dried (over three hours at 37° C) after each of the different steps in the iron hematoxylin procedure. After each interruption the slides were placed in the next solution and the staining was completed in the usual manner. Only those slides were unsatisfactory which were dried after removal from the graded alcohols during dehydration. Therefore, contrary to the usual direction never to allow fecal smears to dry during the staining procedure, in the PVA fixative technique no particular harm is done at most of the steps.

Polyvinyl alcohol is marketed under the trade name of Elvanol in various grades representing degrees of polymerization and hydrolysis. The product used in this study was Elvanol 90-30 obtained from E. I. du Pont de Nemours and Co. Electrochemicals Department, Wilmington 98, Del. This does not represent an endorsement of the product by the Public Health Service.

## OBSERVATIONS AND DISCUSSION

No differences have been observed between organisms stained after preservation with PVA-fixative and those stained after usual methods of Schaudinn fixation. Nuclear details and cytoplasmic inclusions are clearly defined. Rapid and complete fixation apparently occurs, since many trophozoites exhibit protruding pseudopods.

One distinct advantage of PVA-fixative is that it makes possible successful staining of organisms in liquid specimens. Anyone who has ever attempted to prepare permanent stained mounts of protozoa in cultures, watery stools, or pond water has experienced the difficulty of retaining organisms on the slide during staining procedures. When PVA-fixative is used, it serves to adhere as well as to preserve the organisms and prevents their loss during staining. Stained slides containing many organisms have been made from the following liquid specimens: diarrhetic stools and liquid overlay of cultures containing trophozoites of *E. histolytica* (Figs 1 and 2), loose stools containing trophozoites of *E. coli* (Fig 3), *Iodamoeba buetschlii* (Fig 4), *E. nana* (Fig 5), *D. fragilis* (Fig 6), and *G. lamblia* (Fig 7), cultures and pond water containing *Paramecium* (Fig 8) and other protozoa, and tap water containing miracidia of *Schistosoma mansoni* (Fig 9). The only other way known to the authors to stain consistently large numbers of organisms in such specimens is the bulk centrifuge method employing test tubes. However, the PVA-fixative technique is considerably simpler, particularly when only a few slides are to be prepared.

The technique is also useful for preserving interesting specimens or teaching materials for subsequent staining. A variety of organisms have been preserved in liquid fixative and in dried films for about a year without showing any changes when stained. Of particular interest is a specimen of aspirated material from an amoebic lung abscess preserved in a vial with PVA fixative (Fig 10).

At present PVA-fixative cannot be recommended unreservedly for preserving specimens containing cysts. Larger cysts are frequently distorted, apparently during drying of the PVA film. However, in specimens containing many cysts, enough of them with distinct characteristics are usually present to insure correct identification. Occasionally, exceptionally good preparations are obtained even of *E. coli* (Fig 11) and *I. buetschlii* (Fig 12) cysts. Smaller cysts such as *G. lamblia* (Fig 13) and *E. nana* (Fig 14) are usually not adversely affected. As would be expected, staining PVA-fixative films is not an efficient technique for diagnosing helminth eggs.

In general, stool specimens containing cysts and eggs are best examined in wet mounts prepared either directly from the specimens or following a concentration procedure. In such mounts the refractive appearance of unstained cysts and eggs makes them relatively easy to detect with a low-power objective. Helminth eggs and some cysts usually can be diagnosed in temporary mounts of PVA-fixed specimens, but the fixative destroys the refractiveness of the organisms and renders them more difficult to find and identify.

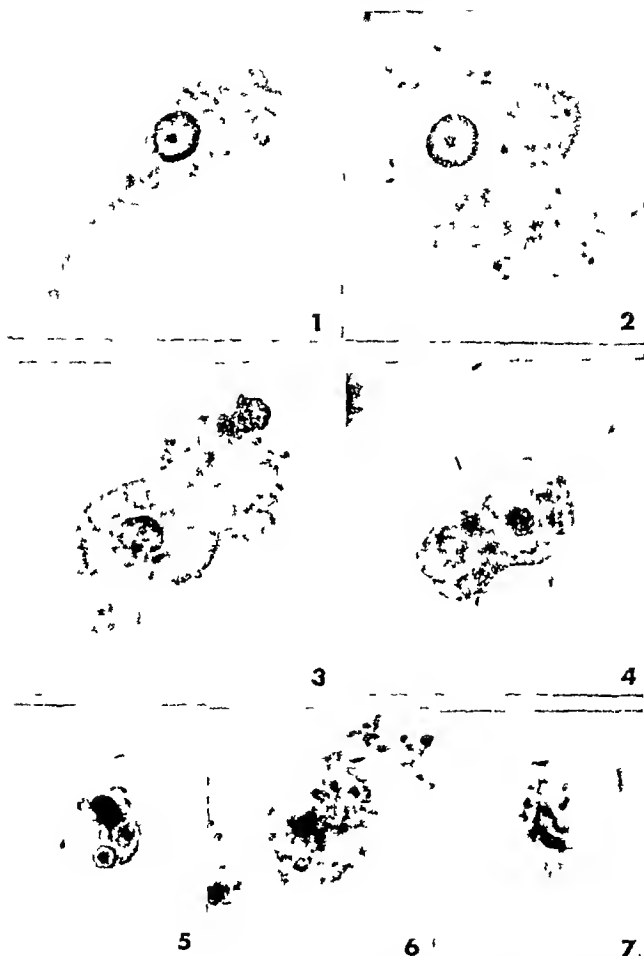


Fig 1—*L. histolytica* trophozoite from diarrheic stool. Note protruding pseudopodia

Fig 2—*L. histolytica* trophozoite from liquid overlay of a culture.

Fig 3—*E. coli* trophozoite from loose stool

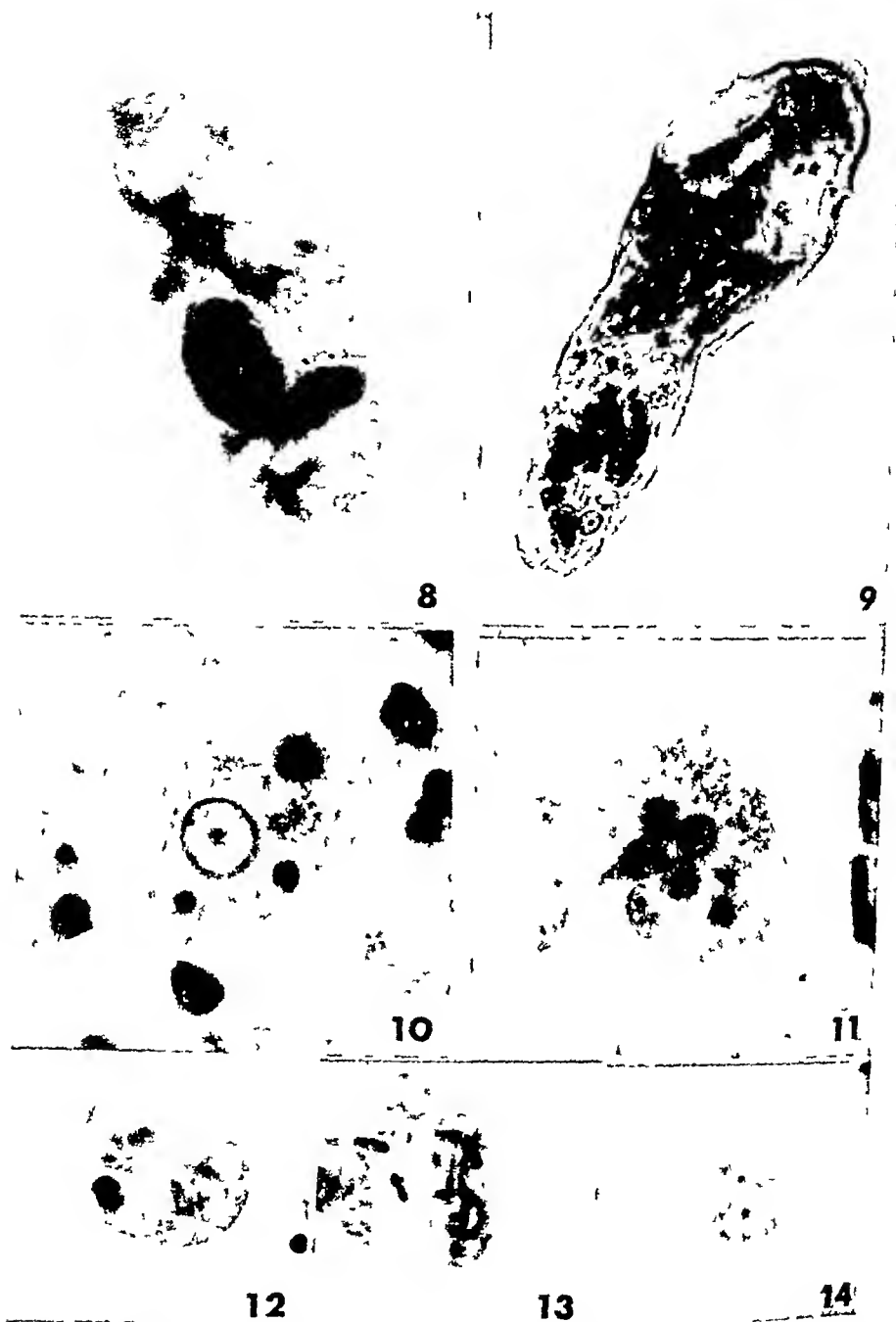
Fig 4—*J. baetschii* trophozoite from loose stool

Fig 5—*E. nana* trophozoite from loose stool

Fig 6—*D. fragilis* trophozoite from loose stool

Fig 7—*G. lamblia* trophozoite from loose stool

(All photographs are of organisms stained in PVA fixative films. Except as indicated films were stained by the long Heldenhaien iron hematoxylin procedure and were photographed at a magnification of 115 diameter.)



- Fig 8—*Paramecium* sp from hay infusion culture Note micronuclei (DeLafeld's hematoxylin  $\times 500$ )
- Fig 9—*S. mansoni* miracidium from tap water (Iron hematoxylin [Goldman, 1949]  $\times 500$ )
- Fig 10—*F. histolytica* trophozoite from lung abscess
- Fig 11—*E. coli* cysts from stool Weigert's copper chrome procedure
- Fig 12—*I. buetschlii* cyst from stool
- Fig 13—*G. lamblia* cyst from stool
- Fig 14—*E. nana* cyst from stool
- (All photographs are of organisms stained in PVA-fixative films. Except as indicated films were stained by the long Heidenhain iron-hematoxylin procedure and were photographed at a magnification of 1125 diameters.)



Although the PVA fixative technique might be applied to the study of many different types of organisms, it has been developed primarily to assist the public health laboratory in securing diarrheic, dysenteric, purged, and protozoan specimens in a satisfactory condition for diagnosis of amoebiasis. The methods described in this report can be adapted easily to this need. For submitting stool specimens small vials (15 ml capacity), two thirds full of PVA fixative, can be distributed to physicians in the same manner that vials containing other preservatives are supplied. Since the PVA fixative technique is not a good method for diagnosing cysts and eggs, the physician should also be supplied with a vial containing no preservative. He should be instructed to place approximately 4 ml portions of *fresh* liquid or formed stool in each vial and to mix the specimen thoroughly in the vial containing fixative.

With the two vial method of submitting specimens, a variety of parasitological techniques can be performed and demonstrations of protozoa and helminths can be made regardless of their stage of development. For example, the vial containing PVA fixative will furnish material for many smears which can be stained and examined for trophozoites and cysts of amoebae and other intestinal protozoa. The vial containing no preservative will furnish material for such gross examinations as consistency of stool, blood, mucus, portions of adult worms, etc. for microscopic examination of temporary mounts (saline and iodine) for protozoan cysts and helminth eggs and larvae and for concentration procedures for cysts and eggs.

The expense involved in preparing PVA fixative is nominal. One pound of PVA costs less than a dollar and is sufficient to make enough fixative to fill a thousand 15 ml vials approximately two thirds full. Other ingredients of the fixative—mercuric chloride, ethyl alcohol, acetic acid and glycerine—are standard laboratory reagents.

#### SUMMARY

1 Methods are described for the use of polyvinyl alcohol (PVA) fixative as a preservative for intestinal protozoa and other parasitic and free living organisms.

2 Organisms so preserved will remain suitable for staining for several months. Best results are obtained with amoebic trophozoites which are otherwise difficult to preserve.

3 In addition to its preservative action, PVA fixative acts as an adhesive to prevent loss of organisms from smears during staining procedures.

4 The technique has been developed primarily to aid in the diagnosis of amoebiasis and is readily adapted to this need. A two vial method for shipping stool specimens is suggested which would enable the laboratory to recover all diagnostic stages of the intestinal parasites.

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## DESIGN OF A PUMP SUITABLE FOR BLOOD

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THERE has been a need in experimental medicine and therapeutics for a pump that could convey blood under sterile conditions without hemolysis, acceleration of the clotting process or introduction of pyrogens. One special interest has been in a pump which will bring the blood of a human subject from a vein to a dialysis apparatus for the removal of products of uremia at variable and appropriate rates. Recently, a number of types of extracorporeal blood dialyzers have been made some of which utilize pumps of various designs. However, none of these completely satisfies all of the above mentioned requirements. The various dialyzing procedures for the treatment of uremia in relation to dietary and other measures have been the subject of a comprehensive review by Snapper<sup>1</sup>. De Leeuw and Blaustein<sup>2</sup> and Vanatta, Muirhead, and Grollman<sup>3</sup> have noted that the Beck pump which forms an integral part of the original Kolff 'artificial kidney' and serves to return blood from the dialyzer to the vein of the patient itself produces hemolysis of the blood circulating through it. Vanatta and co-workers have eliminated, therefore, the use of the pump for this purpose and were able to substitute gravity for the return of the blood.

In the following report a simple pump will be described that meets our stringent requirements rather well. In this apparatus there are no valves, close fitting or metal parts in contact with the blood. Dead spaces where the current moves slowly are eliminated and air bubbles are not introduced. A self-regulating pressure principle provides safety for the cellophane tubing in the dialysis apparatus proper and sterility is accomplished by autoclaving.

In principle the pump consists of a Tygon tube of suitable wall thickness and diameter which is compressed in a wave like motion by a series of twelve keys that push the column of fluid ahead. Behind the crest of the wave the plastic tubing expands by its own resiliency to fill up again. The baffle plate against which the tubing is compressed is adjusted so that the compression of the tubing is incomplete. Pumping will then cease at a predetermined pressure if increased resistance in the outer circuit causes that pressure to be attained. Furthermore the compression of the tubing is largely performed by one elevated edge of the key rather than by a flat surface, reducing thereby the area in which mechanical trauma may occur.

In the apparatus (Fig. 1) a constant speed electric motor with a gear device drives a screw shaped series of twelve rings on an axle. The rings elevate a series of twelve keys to create pressure on the tubing. The rings are evenly spaced at intervals to form one complete sine wave. The amplitude of the wave can be limited by restricting the extent of motion of the keys. The

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tubing lies between the keys and the adjustable plastic baffle. In usage, the tubing is autoclaved and placed on the keys and the baffle is fastened on top. The baffle can be set for complete compression of the tubing, with the attainment of pressures well over 300 mm Hg. By loosening the set screws, the baffle can be adjusted to allow leakage at any intermediate pressure.

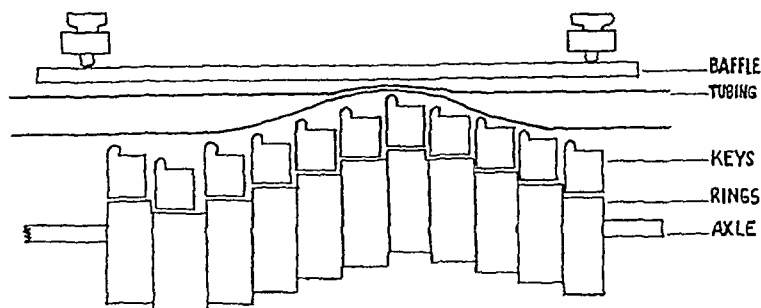


Fig 1—Cross-section diagram of pump. Compression incomplete at height of wave

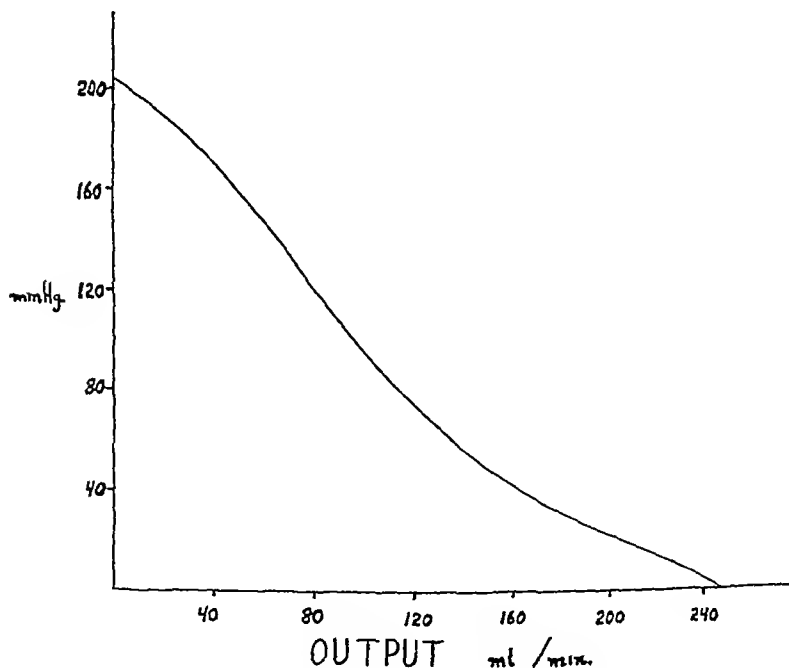


Fig 2—Relationship between output and pressure within the system

In Fig 2 the inverse relationship between output and pressure within the system is shown in an experiment in which the baffle was regulated to have pumping stop at about 200 mm Hg. The increase in pressure within the output or arterial side of the system is attained by increased peripheral resistance. Increased resistance on the suction or venous side of the pump would have the same effect of lowering the output of the pump. By limiting the maximal pressure, the cellophane tubing used in dialysis can be safely guarded against undue stress which can result in breakage.

Outputs of from 1 to 18 liters per hour were obtained by varying the stroke volume and the diameter of the tubing used in the model at hand. The output remained constant as long as the peripheral resistance was unchanged.

The pump in the Kolff "artificial kidney" has rollers that create shearing stresses in the tubing and provide a large area for mechanical trauma. In the apparatus described in this report no shearing motion is possible since the keys can move only in the vertical plane. The rate of hemolysis of the blood recirculated through a closed circle of tubing of 20 ml<sup>3</sup> volume at 37° C was determined with the pump herein described. Since hemolysis was not observed with normal blood, freshly drawn heparinized blood from a patient in uremia (urea N 148 mg per cent) was taken for testing. The rate of blood flow was 125 ml per minute and the maximum pressure attainable was set at 220 mm Hg. Absolutely no hemolysis was discernible after 12 minutes (72 circuits of the blood). In 18 minutes of recirculation of the blood (108 circuits) a very faint trace and in 24 minutes (144 circuits) a faint trace of hemolysis appeared. Since about 50 circuits of the blood volume through our pump and dialyzer is the maximum recirculation to be expected the mechanical trauma to the blood by the pump is negligible.

In summary, a pump is described that conveys blood under sterile precautions without hemolysis for 72 circuits of the blood volume. It is without valves, contains no dead space and provides safety when coupled to a cellophane tube dialyzer by ceasing to pump at predetermined pressures.

The cooperation of Mr G J Frank, Jr and Mr J C Marsh of the Marsh Laboratory, Pittsburgh, Pa., is gratefully acknowledged.

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# AN ELECTROMETRIC METHOD FOR THE DETERMINATION OF RED BLOOD CELL AND PLASMA CHOLINESTERASE ACTIVITY

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THE method for the determination of cholinesterase activity described in this paper was developed to provide a more convenient procedure than those used previously, and one which would require only apparatus generally available in the average laboratory rather than the specialized apparatus heretofore necessary

The principle of the method is fundamentally the same as that used in all of the chemical methods that have been described and depends upon measurement of the acid produced by the action of cholinesterase on acetylcholine. The acid production is measured in terms of the change in pH produced by enzymatic activity in a standard buffer solution over a definite period of time. The pH is measured with the glass electrode.

The rate of pH change will be a satisfactory measure of enzyme activity if an approximately linear relationship between these two quantities can be established. It is well known that cholinesterase activity decreases markedly with decrease in pH over the range from pH 8 to pH 6, which is approximately the range covered in this method. A buffer has been chosen therefore whose decrease in buffer capacity over this range closely approximates the decrease in enzymatic activity. A further consideration is the possible effect of the added enzyme source (plasma or red cells) on the total buffer capacity of the system. This has been minimized by sufficient dilution of the plasma or red cells so that any buffering effect is low compared with the total buffer present.

The data on which the method is based have been obtained using human blood, but the method is applicable in principle to the blood of other species, and to other tissues, provided that proper allowance is made for the buffer capacity of the material being used.

## EXPERIMENTAL

### Reagents Required —

1 *Buffer I (for Red Cells)* 0.02M sodium barbitol (4.1236 Gm.), 0.004M  $\text{KH}_2\text{PO}_4$  (0.5446 Gm.), 0.60M KCl (44.730 Gm.) For 1 liter of buffer the reagents are dissolved in 900 ml of distilled water, 28.0 ml of 0.1N HCl are added while shaking the solution, and the volume is then made to the mark. The pH of buffer I should be 8.10 at 25° C.

2 *Buffer II (for Plasma)* 0.006M sodium barbitol (1.2371 Gm.), 0.001M  $\text{KH}_2\text{PO}_4$  (0.1361 Gm.), 0.30M NaCl (17.535 Gm.) For 1 liter of buffer dissolve the reagents in about 900 ml of distilled water and add 11.6 ml of 0.1N HCl before diluting to volume. The pH of buffer II should be 8.00 at 25° C.

3 *Acetylcholine Substrate for Red Cells* 0.11M acetylcholine chloride (2.000 Gm in 100 ml of distilled water)

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✓ 4 *Acetylcholine Substrate for Plasma* 0.16*M* acetylcholine chloride (3.000 Gm in 100 ml of distilled water)

A few drops of toluene are added to all the foregoing solutions as a preservative and the solutions are kept in a refrigerator when not in use

5 0.01 per cent saponin

6 0.90 per cent NaCl

These two solutions are made fresh as needed

*Method for Red Cells*—Approximately 5 ml of freshly drawn blood, containing sufficient heparin to prevent clotting are centrifuged at 2,000 r.p.m. for fifteen minutes in a graduated centrifuge tube and the plasma is separated. The cells are mixed with 2 to 3 vol of 0.9 per cent NaCl and again centrifuged at 2,000 r.p.m. for fifteen minutes. After discarding the supernatant fluid the operation is repeated, centrifuging this time for twenty minutes. The volume of the cells is noted and then the saline supernatant is removed to the point where the remaining volume of cells and saline is twice the volume of cells alone. The cells are then mixed thoroughly with the remaining saline. After mixing 0.4 ml of cell suspension is hemolyzed in 9.6 ml of 0.01 per cent saponin solution. One milliliter of hemolyzed red cell solution, representing 0.02 ml of cells is added to 1 ml of buffer solution I and placed in a water bath at 25° C. After allowing ten minutes for equilibration, the initial pH ( $pH_i$ ) is determined with a pH meter reading to the nearest 0.01 pH unit. Then 0.2 ml of 0.11*M* acetylcholine solution is added with rapid mixing and the time is recorded. The enzymatic reaction is allowed to proceed for one to one and one half hours and then the final pH of the solution ( $pH_f$ ) is determined. It is necessary to shake the beaker containing the reaction mixture a few seconds after the electrodes have been immersed to establish rapid equilibrium. The time at which the pH was measured is recorded, and the cholinesterase activity in units of  $\Delta pH/\text{hour}$  is calculated by use of the following formula

$$\Delta pH/\text{hour} = \left( \frac{pH_f - pH_i}{t_f - t_i} - b \right) f \quad (\text{Equation 1})$$

where  $pH_i$  = initial pH

$pH_f$  = final pH

$t_i$  = time of mixing with acetylcholine

$t_f$  = time of reading pH

$b$  = nonenzymatic hydrolysis correction corresponding to pH

$f$  = correction for variations in  $\Delta pH/\text{hour}$  with pH corresponding to pH

The  $b$  and  $f$  corrections are given in Table I

TABLE I CORRECTION FACTORS FOR USE IN EQUATION 1

pH	RED CELL CHOLINESTERASE CORRECTIONS		PLASMA CHOLINESTERASE CORRECTIONS	
	$b$	$f$	$b$	$f$
7.9	0.03	0.94	0.09	0.95
7.8	0.02	0.95	0.07	1.00
7.7	0.01	0.96	0.00	1.01
7.0	0.00	0.97	0.05	1.02
7.5	0.00	0.98	0.04	1.02
7.4	0.00	0.99	0.03	1.01
7.3	0.00	1.00	0.02	1.01
7.2	0.00	1.00	0.01	1.00
7.1	0.00	1.00	0.02	1.00
7.0	0.00	1.00	0.01	1.00
6.8	0.00	0.99	0.01	1.00
6.6	0.00	0.97	0.01	1.01
6.4	0.00	0.94	0.01	1.01
6.2	0.00	0.97	0.01	1.04
6.0	0.00	0.99	0.01	1.09

This volume is dependent in part upon the centrifugal force and the time during which it is applied. The average centrifugal force used in this work was approximately 800 times gravity.

To express results in terms of per cent activity relative to some normal value, multiply the  $\Delta$  pH/hour value found for the sample by 100 and divide by the  $\Delta$  pH/hour value considered to be (or found to be) normal. For example, the average  $\Delta$  pH/hour value for red cell cholinesterase activity in twelve normal human subjects has been found to be 0.753. If an unknown sample gave a value of say 0.550, then the per cent activity would be

$$\frac{0.550}{0.753} \times 100 = 73 \text{ per cent}$$

The red cell buffer (buffer I) is made to give a pH of 8.00 after mixing with diluted red cells. Sodium barbital has its maximum buffer capacity in the region of pH 8.0<sup>1</sup> and this is also the region of optimal cholinesterase activity\*. With the ratio of buffer to red cells used, the decrease in buffer capacity parallels the decrease in enzyme activity with decreasing pH to give an almost constant value for the enzyme activity in  $\Delta$  pH/hour. This is shown by the relatively small deviations from unity of the  $f$  factors given in Table I. Potassium dihydrogen phosphate is added to maintain the nearly constant relationship down to pH 6.00. The buffer capacity contributed by the added red cells averages 12 per cent of the total buffer capacity, determined by titration between pH 8.00 and pH 7.00.

The activating effect of potassium chloride on red cell cholinesterase has been described by Mendel and Rudney<sup>3</sup> and Augustinsson.<sup>2</sup> This salt was used in preference to the magnesium or calcium salts customarily employed as activators to avoid precipitation of phosphate. The concentration employed was found to be in the optimum range for activation. Hemolysis by saponin prevents the precipitation of the red cell stroma by the potassium chloride in the buffer.

Although the substrate concentration is greater than the optimum reported for mammalian red cell cholinesterase by Augustinsson,<sup>2</sup> it was necessary to use a final concentration of 0.01M acetylcholine in order to maintain the nearly constant rate of pH change over the selected pH range.

*Method for Plasma*—The plasma is diluted with water so that each milliliter of solution contains 0.02 ml plasma. One milliliter of diluted plasma is mixed with 1 ml of buffer solution II. The solution is allowed to equilibrate in a water bath at 25° C for ten minutes, and then 0.2 ml of 0.165M acetylcholine is added with mixing. The subsequent determination is carried out, and the activity calculated, in the same manner as described for red cells, using the correction values for plasma given in Table I.

Human plasma contains less cholinesterase activity and has less buffer capacity than the equivalent volume of red cells. Therefore, a more dilute buffer is used. However, similar considerations apply to the decrease in enzyme activity with decreasing pH, as discussed in the preceding section. The buffer capacity of the plasma averages 7 per cent of the total. Salts have a relatively small effect on the activity of human plasma cholinesterase,<sup>4</sup> but the pH of the dilute buffer used can be affected by changes in salt concentration. Sodium chloride is added to the buffer to reduce possible variations in total salt concentration.

Both buffers I and II slowly decrease in pH and buffer capacity after several weeks and should be checked with a pH meter at the time they are used. If the pH<sub>i</sub> reading for a determination is more than 0.03 pH units below 8.00, a fresh buffer solution should be prepared.

Pure acetylcholine salts will produce a pH change of less than 0.01 pH unit when added to buffers I or II in the concentrations specified in the Methods sections.

*Comparison of the Electrometric Method With the Manometric Method*—Cholinesterase was determined on thirty-one independent samples of plasma and twenty-seven independent samples of red cells from twelve male human subjects, using both the electrometric method described here and a modified form of the manometric method of Ammon.<sup>6</sup> In the manometric procedure the reaction mixture for plasma cholinesterase contained 0.05 ml of plasma and 3.0 ml of 0.025M sodium bicarbonate. For determination of red cell cholinesterase

\*See reference 2 for bibliography.



each Warburg manometric vessel contained 0.02 ml of cells and 3.0 ml of 0.025M sodium bicarbonate containing 0.0342M magnesium chloride. In each instance the substrate was recrystallized acetylcholine bromide in a final concentration of 0.015M. The enzyme was tipped from the side arm after ten minutes of equilibration with 5 per cent carbon dioxide in nitrogen. Manometric readings were taken over a forty minute period, the temperature being maintained at 38° C. The electrometric procedure was carried out as described here, the temperature of the reaction being 25° C.

The mean value of the cholinesterase activity by the electrometric method was 0.703  $\Delta$  pH per hour for plasma and 0.753  $\Delta$  pH per hour for red cells. The corresponding values by the manometric method were 226.0 microliters per hour for plasma and 253.0 microliters per hour for red cells. Relative activities were calculated for both methods by dividing individual activities by the appropriate mean values. The differences between the relative activities determined by the electrometric method and the corresponding relative activities by the manometric method were calculated. The standard deviation of the differences was 5.49 per cent for plasma cholinesterase and 5.50 per cent for red cell cholinesterase.

*Nonenzymatic Hydrolysis Correction*—The nonenzymatic hydrolysis correction,  $b$ , was determined by measuring the pH change with time in solutions containing buffer, acetylcholine, and inhibited cholinesterase. Diisopropyl fluorophosphate (DFP) (10  $\mu$ g per milliliter of diluted cells or plasma) was used to inhibit the cholinesterase. The pH was determined at intervals of several hours and  $b$  was then calculated by subtracting the observed pH from 8.00 and dividing by the time in hours.

A slow lowering of pH occurs in the solution of the buffer and enzyme in the absence of substrate and is included in the  $b$  correction.

*Correction Factor  $f$* —The values for the correction  $f$ , given in Table I, are based on samples taken from ten subjects. To determine the values, activity measurements were made as described in the Methods section, but pH readings were taken successively on each sample at approximately ten minute intervals over the pH range from 8.00 to 6.00. The value of  $f$  at pH 7.00 was arbitrarily set equal to 1.00. Using Equation 1, the values of  $f$  were calculated for the selected pH range.

#### DISCUSSION

The advantages of the electrometric pH method are simplicity, a requirement for a minimum of equipment, and the possibility of doing a large number of determinations in a relatively short time. The pH readings may be made easily in a time of one minute for each sample. It is therefore possible to do a large series of determinations in a few hours by adding the substrate to the samples at one minute intervals.

After this method had been developed, a similar procedure by Cioatto and associates<sup>7</sup> was found in which rate of pH change is used as a measure of serum cholinesterase activity, the pH being measured colorimetrically in a Pulfrich photometer. This method is applicable only to relatively uncolored solutions.

## SUMMARY

1 An electrometric method for plasma and red cell cholinesterase activity is presented in which the enzymatic release of acetic acid from acetylcholine is measured in units of pH change per hour, in a solution of standard buffer capacity

2 The method has been compared with the standard manometric procedure. The standard deviation of the differences between the methods was 5.49 per cent for plasma cholinesterase and 5.50 per cent for red blood cell cholinesterase.

The author wishes to thank Mr. P. Zirnbliis and Mrs. P. D. McNamara for their assistance in this work.

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# STERILIZATION OF DIFUNCTIONALIZED LOOPS OF COLON IN PREPARATION FOR ANASTOMOSIS WITH OTHER VISCERA

## A METHOD OF STUDY AND APPROPRIATE SELECTION OF ANTIBACTERIAL AGENTS

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IN ADMINISTERING intestinal antiseptics for surgery of the large bowel a reduction of the bacterial count of the feces timed to be at a maximum on the day of operation is desirable. Two patients were recently treated with a combination of antibiotic and chemotherapeutic agents to prepare the colon for anastomosis with other viscera. At operation full thickness segments of colon approximately 10 cm. square were excised from the operative site for aerobic and anaerobic bacteriologic cultures. In the first case the cultures revealed no growth. Forty-eight hour cultures from the second case were sterile, but at the end of seventy-two hours rare colonies of *Monilia albicans* and diphtheroid bacilli were recovered. All organisms in the pretreatment cultures of colonic contents which showed the usual profuse mixed intestinal flora were absent (Table I).

The first patient (R. I.) was a 61-year-old man with recurrent obstructing carcinoma of the esophagus following a previous transthoracic resection of which time an extremely short jejunal mesentery was noted. After preliminary gastrostomy for the obstruction, a rigid transverse colostomy was done to facilitate the preparation of his bowel. On April 13, 1949, Dr. R. H. Smithwick did a transthoracic resection of the recurrent carcinoma and because the short jejunal mesentery precluded an esophagojejunostomy the transversocolon was used for an esophagocolostomy. At a second stage the continuity of the gastrointestinal tract was re-established, leaving a transplanted segment of colon within the thoracic cavity.

The second patient (W. O.) was a 64-year-old man with carcinoma of the anterior wall of the rectum. During a preliminary cystoscopy the instrument was inadvertently passed through the posterior urethra into the rectum through the carcinomatous mass. Because of this a transverse colostomy was done the same day and it later proved useful in preparing the bowel for surgery. On May 26, 1949, a resection of the rectum, bladder, prostate, seminal vesicles, and posterior urethra with ureteroenteric anastomosis was done.

Both of these cases will be reported fully at a later date but the unexpected bacteriologic findings have prompted this preliminary report on the method used for study and preparation of the bowel.

### PLAN OF STUDY AND ANTIBACTERIAL TREATMENT

Therapy was planned to secure reduction in the flora of the colon, timed to be maximal on the day of operation. The choice of antibiotic agents was guided by the sensitivity of the intestinal flora in each instance. Cultures taken just before operation were used to work out the sensitivities to various antibiotics of the organisms remaining in the colon as changed by the intes-

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TABLE I PREOPERATIVE AND POSTOPERATIVE CULTURES, INTESTINAL FLORA, AND COLIFORM COUNTS

	ORGANISMS	INITIAL CULTURE					PREOPERATIVE CULTURE	FINAL CULTURE (EXCISED COLON TISSUE)				
Case 1 (Patient R I)	<i>Staphylococcus albus</i>	+					0	0				
	<i>Diphtheroid bacilli</i>	+					+	0				
	<i>Nonhemolytic streptococcus</i>	+					+	0				
	<i>Alpha hemolytic streptococcus</i>	+					+	0				
	<i>Bacillus mucosus capsulatus</i>	+					0	0				
	<i>Escherichia coli</i>	+					0	0				
	<i>Aerobacter aerogenes</i>	+					+	0				
	<i>Bacillus pyocyaneus</i>	+					+	0				
Preoperative day		10	9	8	7	6	5	4	3	2	1	
Coliform count/gram of feces		10,000					0					0
Case 2 (Patient W O)	<i>Staphylococcus albus</i>	+					+	0				
	<i>Alpha hemolytic streptococcus</i>	+					0	0				
	<i>Alpha hemolytic streptococcus (enterococcus type)</i>	+					+	0				
	<i>Bacillus pyocyaneus</i>	+					+	0				
	<i>Escherichia coli</i>	+					0	0				
	<i>Proteus vulgaris</i>	+					0	0				
	<i>Bacillus mucosus capsulatus</i>	+					0	0				
	<i>Monilia albicans</i>						0	+				
Preoperative day		13	12	10	9	8	7	6	3	2	0	
Coliform count/gram of feces		10,000		12,000					30,000		5,500	

tinal antiseptic therapy. Aimed with this information in advance, it should be possible to give systemic or topical treatment with greater speed and specificity for an infection which might develop from the colon after operation. Because of the rapid development of resistance of certain organisms to streptomycin, it was decided to refrain from using it as an intestinal antiseptic but to reserve it for later use in case infection developed.

Cultures of colon contents were obtained through the distal limb of the colostomy. A sterile rubber tube was inserted as far into the bowel as possible and a long slender pipette passed through this tube was used to aspirate colonic juice. All cultures were planted on blood agar plates and inoculated into 60 cc of beef heart infusion broth enriched with sheeps' blood and fortified with 50 mg per cent of paraaminobenzoic acid, each inoculation consisting of a 2 mm platinum wire loopful of the juice. Tubes of chopped meat media fortified with infusion broth and sealed with petroleum jelly and paraffin were used for anaerobic cultures. Coliform counts were done by a modification of the streak method described by Poth.<sup>1</sup>

Cultures of feces were taken on the day the colostomies were opened and are shown in Table I. Colostomy and rectal enemas and instillations of 25 per cent magnesium sulfate served to clean out the isolated loops of colon. Patients were given a low residue diet and vitamin K parenterally.

In order to get a rough idea of the sensitivity of the intestinal flora upon which to base antibiotic therapy, blood agar plates were inoculated with swab cultures of the feces and tested by a filter paper disk contact method for sensitivity to aureomycin and chloromycetin.\*

\*A discussion of this method will be the subject of a separate report.

Twenty four hour broth cultures diluted  $10^6$  were also tested by a serial dilution technique against these same two antibiotics with the idea of using the most appropriate one as indicated by these sensitivities. The chosen antibiotic was given together with Sulfathaladine as an intestinal antiseptic. It was hoped that this combination would be effective against both gram negative and gram positive organisms. The results of these and the subsequent sensitivity tests are indicated in Table II. In the presence of an enterostomy or diarrhea or when enemas or purgatives are to be used Sulfathaladine is preferable to Sulfasuxidine because of its greater bacteriostatic activity; and therefore it was chosen in these cases. For the first patient (R I) chloromycetin was selected rather than aureomycin because, although the flora was within range of sensitivity to both antibiotics aureomycin would have been more likely to cause nausea and the patient was in precarious fluid and electrolyte balance. Inasmuch as chloromycetin was also within the effective range of sensitivity it was selected to be given with the Sulfathaladine. In the second patient (W O) the flora was within the range of sensitivity to both antibiotics so aureomycin was selected because it was more readily available. Administration of the chosen agents was then started via all available routes. Large doses were given as colostomy and rectal irrigations and also because of the possibility of spill over contamination from the proximal colostomy loop these drugs were given through the gastrostomy and by mouth as well. Table III indicates the dosage and routes of administration of the various agents.

On the third day before operation cultures from the colonic juice were obtained to work out the sensitivities of each remaining organism as changed by a period of therapy in order to better cope with infection should it occur (Table II).

On the second or first preoperative day it was desired to administer prophylactic penicillin and since Poth and co-workers have shown a definite penicillin Sulfathaladine antagonism affecting certain gram negative organisms the Sulfathaladine was discontinued and Sulfasuxidine was substituted. This allowed the unimpeded action of penicillin for the last one or two days before operation after having taken advantage of the superior action of Sulfathaladine during the major part of the preparation. Intramuscular penicillin was then started, and to the first patient (R I) penicillin aerosol and lozenges also were given in an attempt to cut down the gram positive flora in the nasopharynx, throat and esophagus.

The daily dose of Sulfathaladine and Sulfasuxidine was crushed and suspended in saline in which the antibiotic had been dissolved. This was then instilled into the colostomy, gastrostomy and rectum in equally divided doses at 6 and 10 A.M. and 2 P.M. and 10 P.M., the volume of each instillation being about 100 to 200 cubic centimeters. The last day's dose was dispensed in a very small volume (10 cc. for each instillation) so that the bowel would be dry at operation. Oral medications were given in equally divided doses on the same schedule.

The following is a summary of the plan of study and treatment in outline form:

- 1 Reserve streptomycin for later use if infection should develop.
- 2 After the colostomy is open culture the transverse colon. Test the mixed cultures for sensitivity against available antibiotics suitable for oral administration.
- 3 Give colostomy and rectal enemas and magnesium sulfate instillations until the bowel is clean then use appropriate antibiotic agent along with Sulfathaladine for instillations into the bowel.
- 4 Three days before operation culture the mid colon and work out sensitivities of the constituents of the flora as now altered by therapy to various antibiotics.
- 5 Two days before operation omit Sulfathaladine and substitute Sulfasuxidine. Start intramuscular penicillin, penicillin aerosol and penicillin lozenges.

TABLE II. SENSITIVITY TEST RESULTS

CASE	ANTIBACTERIAL AGENT TESTED	INITIAL MIXED CULTURE		PRELIMINARY CULTURE—INDIVIDUAL CONSTITUENTS									
				SPECIAL DILUTION METHOD			FIFTY FIFTY DISK METHOD						
		SERIAL DILUTION METHOD	FILTER PAPER METHOD*										
				L 110 CYNIFLS	A ALPO GINES	NONHMO IATIC SIRTTO COCCUS	ALPHA STREPTO COCCUS	ALPHA STREPTO COCCUS	B 110 CYNIFLS	A PRO GLNFS	NON STREPTO COCCUS	ALPHA STREPTO COCCUS	ALPHA STREPTO COCCUS
1 (R I)	Aureomycin	γ/ml		10	3	30	0.1	0.1	300	1,000	250	500	0.125
	Chloromycetin	γ/ml		25	6.25	6.25	6.25	6.25	50	50	100	50	100
	Streptomycin	U/ml		15	7.5	15	7.5	7.5	0.20	0.20	250	500	100
	Penicillin	U/ml		1,000	250	15	35	35	1,000	1,000	20	100	0.20
	Bactracin	U/ml		1,000	125	>0.3	>0.3	>0.3	>1,000	500	1,000	0.20	0.20
	Sulfamylon	%		0.3	0.6	0.15	0.07	0.07	0.06	1.25	2.5	0.06	0.06
	Urethane	%		50	50	50	50	50	20	20	20	20	---
	Urethane	%		25	25	0.01	0.007	0.007	10	10	0.125	0.125	0.06
	Penicillin	U/ml		250	250	125	0.7	0.7	1,000	1,000	0.125	0.125	0.20
	Streptomycin	U/ml		15	60	30	15	15	0.25	0.20	0.25	25	30
2 (W O)	Sulfamylon	%		0.03	0.15	0.07	0.003	0.003	0.06	0.06	0.06	0.06	0.125
	Aureomycin	γ/ml		5	125	250	0.7	10	0.1	0.1	7	0.1	500
	Chloromycetin	γ/ml		15	50	100	6.25	3.15	3.15	25	25	50	25
	Streptomycin	U/ml		15	63	32	15	125	20	100	100	500	20
	Penicillin	U/ml		0.15	35	0.07	1,000	1,000	0.2	20	100	0.20	1,000
	Bactracin	U/ml		0.3	0.7	15	500	500	0.20	20	100	100	500
	Sulfamylon	%		0.07	0.01	0.007	2.5	2.5	0.06	0.06	0.06	0.06	2.5
	Urethane	%		5	5	2.5	5	5	25	50	12.5	25	25
	Urethane	%		0.003	5	0.0015	5	5	0.125	0.125	0.125	0.125	10
	Penicillin	U/ml		0.3	500	0.15	500	500	0.125	0.125	0.125	0.125	1,000
3	Streptomycin	U/ml		3	0.3	0.15	100	100	0.25	0.25	0.25	50	100
	Sulfamylon	%		0.07	0.007	0.03	2.5	2.5	0.06	0.06	0.06	0.06	1.25
	Sulfamylon	%		0.07	0.007	0.03	2.5	2.5	0.06	0.06	0.06	0.06	1.25

\*Direct swab inoculation onto blood agar plate. Ten minute contact with disk M4 with testing solution. No preliminary incubation results in two flasks indicate the concentration that bracketed the actual sensitivity which has been determined.

TABLE III DAILY DOSE AND ROUTE OF ADMINISTRATION OF ANTIBIOTIC AL VOLUMES

FLOID EXAMINATION	Dose	8	7	6	5	4	3	2	1	DAY OF OPERATION
By mouth or gastrostomy	Sulfathiazole (Gm)	5								0
	Sulfasuxidine (Gm)									0
	Chloromycetin (Gm)									0
	Sulfathiazole (Gm)	1	1	1	1	1	1	1	1	0
	Sulfasuxidine (Gm)									0
Case 2 (W O)	Sulfathiazole (Gm)	8	8	8*	†	†	†			0
	Sulfasuxidine (Gm)									0
	Aureomycin (Gm)									0
	Crysticillin (units)									300,000
	Penicillin (units)									300,000
I encellin	Penicillin (units)									0
	Penicillin aerosol (units)									0
	Penicillin troches (units)									0
	Crysticillin (units)									0
	Penicillin (units)									0
Case 2 (W O)	Crysticillin (units)	600,000	600,000	600,000						300,000
	Penicillin (units)									600,000
	Sulfathiazole (Gm)	5	8	8	8	8	8	8		0
	Sulfasuxidine (Gm)									0
	Chloromycetin (Gm)		2	2	2	2	2	2		0
Installed into colonostomy or rectum	Sulfathiazole (Gm)									0
	Sulfasuxidine (Gm)	1	1	1						0
	Aureomycin (Gm)	3	3	3*	3	3	3	3	3	0
	Crysticillin (units)									0
	Penicillin (units)									0

Case 1 (Patient W. O.) was being treated with aureomycin, Sulfasuxidine, and procaine penicillin (crysticillin) for the urethral perforation before the sixth operative day when the regime for preparation of the bowel as outlined in the text was begun.

† Given by error.

\* Oral administration omitted because of marked skin irritation around colostomy.

## A TECHNIQUE FOR THE COLLECTION OF LYMPH FROM THE RIGHT THORACIC DUCT IN DOGS

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ST. LOUIS, MO

**S**TUDIES of experimental pulmonary edema have been frequently handicapped by the lack of a method for the detection of edema in intact and functioning lungs, particularly when the transudation was occult or of low intensity. In their studies of pulmonary lymph, Dinker and associates<sup>1</sup> noted that the flow of lymph from the lungs was increased when congestion was induced. We have recently investigated the outflow of lymph from the lungs in relation to congestion and edema of the pulmonary tissue from experimental heart failure.<sup>2</sup> These observations have indicated that the production of lung lymph is always increased with pulmonary engorgement and edema, and that the quantity of flow is greater with more intense grades of transudation.

It has been shown<sup>1</sup> that the pulmonary and cardiac lymph passes through lymph nodes lying along the superior vena cava. The fluid then courses from these nodes to the right subclavian vein through one or more lymphatics comprising the *right thoracic duct*. In some animals, the pulmonary lymphatics pass to the left, anastomosing with the thoracic duct or entering directly into the left subclavian vein. Dinker and co-workers<sup>1</sup> have collected the pulmonary lymph by cannulation of the right thoracic duct. However, the procedure was so difficult as to be "accomplished satisfactorily in about one out of five animals." In our experience, the cannulation technique has had serious drawbacks. In many animals, the lymphatics were found to be too small to cannulate. In others, cannulation was successful but the lymph failed to flow because of mechanical obstruction by the cannula.

We have employed a simple method for the collection and measurement of right thoracic duct lymph which avoids the pitfalls of minute lymphatics and possible artificial obstruction of the vessels by the manipulation.

### METHOD

The procedure is carried out on dogs under Nembutal anesthesia. To facilitate identification of the lymphatic, 10 cc. of 1 per cent solution of Evans blue is instilled into the lower part of the trachea through a glass tube. This tube may be introduced through a tracheotomy or through the glottis. A period of ten minutes is allowed for the absorption of the dye into the pulmonary lymphatics. A longitudinal incision is made along

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the right external jugular vein extending to the pectoral muscles and the first rib. The lower end of the jugular vein is then dissected free and followed to its confluence with the axillary vein and the right subclavian veins (see Fig. 1). The subclavian vein, in its mid portion is in close apposition to the subclavian artery which arches up from beneath. Although there is considerable variation in the right lymphatic vessels, one or more lymphatics have been found in this area constantly, lying either across the ventral surface of the subclavian vein or between the artery and vein arching upward from the deeper tissue. If dissection is first carried out in this field location of the lymphatics requires a very short time; a tedious dissection is usually not necessary.

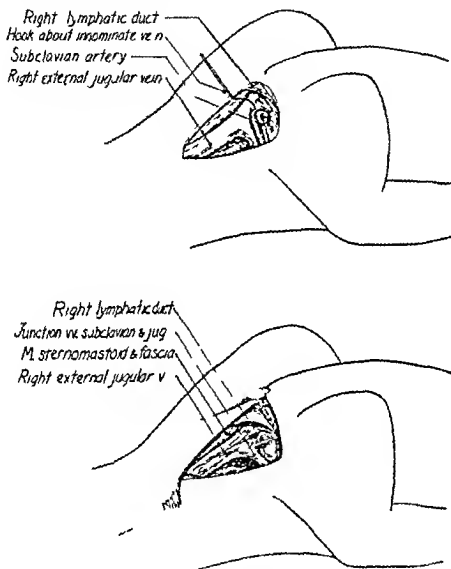


Fig. 1

Collection of the lung lymph is accomplished by incising the vessel with fine scissors permitting the fluid to issue freely. The field is carefully wiped dry and the lymph is then caught on small pledgets of absorbent cotton (10 to 20 mg. in weight) placed over the incised vessel for a measured time. In the properly executed dissection, where all bleeding has been controlled the lymph is the only fluid absorbed by the cotton. Furthermore the identity of the lymph is assured by the bluish stain from the dye. We employ a Roller Smith precision balance to facilitate rapid and accurate weighing of the pledgets.

In normal anesthetized dogs our method shows that the quantity of lymph from a right thoracic lymphatic vessel varies from 4 to 50 mg. a minute. Drinker and co-workers,<sup>1, 2</sup> by cannulation of the lymphatic and the flow to range from 7 to 59.4 mg. a minute. While cannulation is extremely difficult being unsuccessful in four out of five animals, the pledget technique in all but rare instances is easily accomplished.

## SUMMARY

Anatomic landmarks which facilitate the location of the right thoracic duct are described. By incision of the lymphatic vessel, the fluid is collected on weighed cotton pledgets for controlled periods. The method has proved to be accurate and simple to carry out.

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# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty Second Annual Meeting  
Chicago Ill Nov 4 and 5, 1949

## ABSTRACTS

### 1 THE DYNAMICS OF COAGULATION

J GARROTT ALLEN MD PETER V MOULDER MD (BY INVITATION)  
DANIEL M ENERSON, MD (BY INVITATION) AND  
DONALD GLOTZER BS (BY INVITATION)  
CHICAGO ILL

Spontaneous (?) bleeding in the presence of hemophilia prothrombin deficiency and over heparinization is not explained by current knowledge or theories of coagulation. Presumably blood could be extravasated under these conditions because (1) blood is less viscous and its surface tension altered or (2) the continuity of the walls of the small vessels depends upon some continuing contribution from blood which is destroyed when the clotting process is delayed or inhibited.

Measurements of surface tension and viscosity have shown no significant changes in hemophilia prothrombin deficiency or excessive heparinization. Transfusion of normal dogs until hematocrit readings of 65 to 70 per cent were obtained resulted in a considerable increase in viscosity and a decrease in surface tension but these animals bled just as readily from prothrombin deficiency or over heparinization as did untransfused controls. The irradiated dog (450 r) given sufficient blood daily to maintain a hematocrit reading of 70 per cent had similar changes in viscosity and surface tension and developed increased clotting time, thrombocytopenia and hemorrhage. Anemia and hypoproteinemia produced by the daily withdrawal of blood in normal dogs reduced viscosity and increased surface tension but did not cause these animals to bleed or make them unduly susceptible to hemorrhage from Dicumarol or heparin.

Data are presented which suggest that coagulation is a dynamic and protective process. The activity of prothrombin rapidly falls and may disappear entirely fourteen to twenty hours after hepatectomy. Fibrinogen concentrations decline in a similar manner though less rapidly. That the removal of the liver is not necessary for the rapid decline of prothrombin is indicated by the rapid disappearance of transfused prothrombin in whole blood or fresh plasma. Apparently the turnover of prothrombin is rapid and nearly complete in twenty-four hours and that of fibrinogen is only slightly longer (two to three days). The fate of these proteins is not known. It is possible that fibrin is constantly formed and is concerned with the maintenance of the integrity of the vascular wall and that its formation consumes prothrombin.

## 2 SIMULTANEOUS CESAREAN SECTION AND SPLENECTOMY IN IDIOPATHIC THROMBOCYTOPENIC PURPURA

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AND CHAMP LYONS, M D (BY INVITATION)  
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Although splenectomy has been done many times on pregnant patients, we are unable to find any record of simultaneous splenectomy and cesarean section in the management of a complicated pregnancy.

The patient, a 27-year-old white woman, with two preceding spontaneous abortions, was first seen by us in the seventh month of pregnancy, because of purpura. She had a long past history of abnormal bleeding for which no cause had been found. Physical examination showed innumerable petechiae over the legs, scattered purpuric lesions of the rest of the body, and findings compatible with a seven-month pregnancy. Laboratory studies showed an infinitely prolonged bleeding time, platelet count of 2,000 per cubic millimeter, and increased megakaryocytes in the bone marrow. Roentgenogram showed a mild cephalopelvic disproportion.

Because of the significant fetal mortality reported following splenectomy in the third trimester, and because of the relatively minor bleeding, it was decided that the safest course in this patient would be to allow her to try to deliver spontaneously and then perform a splenectomy post partum, early if necessary or later if bleeding was not too excessive. After six hours in labor at term it became apparent that cesarean section would be necessary. A low-flap cesarean section was performed, followed immediately by splenectomy. When the patient entered the operating room the bleeding time was twenty-one minutes, while the abdomen was being closed it was two minutes and thirty seconds. The postoperative course was uneventful and the patient has had no abnormal bleeding for the ensuing thirteen months.

The baby's platelet count at delivery was about 100,000 per cubic millimeter with normal bleeding and clotting times. She is healthy at the age of 13 months, has a normal blood count, and has never had any hemorrhagic phenomena.

The management of this case is not presented as standard treatment for purpura during pregnancy. Rather, it is a hitherto unreported example of the effectiveness of splenectomy in preventing major hemorrhage in a patient with thrombocytopenic purpura in whom another major surgical procedure was necessary as an emergency.

## 3 THE DETECTION OF INTRACRANIAL TUMORS BY THE USE OF DI-iodo<sup>131</sup>-FLUORESCEN

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THEODORE FIELDS, B S (BY INVITATION), AND LOYAL DAVIS, M D  
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Neoplastic tissues have the ability to combine with fluorescent dye. This combination is temporary, lasting for a period of two to four hours after intravenous injection of the dye. When di-iodo<sup>131</sup>-fluorescein (radiofluorescein) in amounts of 0.7 to 1.0 mc is used, the greater radioactivity of the tumor tissue permits one to detect its location by the use of suitably arranged Geiger-Müller counters. This technique has been applied to the diagnostic study of patients with space-filling intracranial lesions. When the lesion is a neoplasm, good

concentration of the radiofluorescein occurs, and the location of the tumor can be established. When the lesion is a nonneoplastic cyst, or hematoma, there is no significant local accumulation of the radioactive material. A description is given of the differential counter method and of the apparatus developed in the Radioisotope Unit, Hines Hospital. The results of the examination of sixty patients with this technique are discussed. In every instance but one, to date, the diagnosis based on the isotope studies has been verified at operation.

#### 4 THE INHIBITION BY NORMAL SYMPATHETIC VASOCONSTRICTOR TONE OF THE SPONTANEOUS DEVELOPMENT OF A COLLATERAL CIRCULATION IN CHRONIC OBLITERATING ARTERIAL DISEASE OF THE LEG

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In 1937 observations were made which suggested that a collateral circulation would spontaneously develop as a normal physiologic response to chronic ischemia of the leg and foot were it not for the fact that the flow of sympathetic vasoconstrictor nervous impulses to the arteries of the leg and foot acted as an inhibiting influence. To test the validity of this premise a series of cases of chronic thromboangitis obliterans and arteriosclerosis obliterans of the lower extremities were subjected to lumbar sympathetic ganglionectomy. During the past eleven years a total of 128 operations has been performed on 112 individuals. On the basis of history, age and physical findings, 32 probably had thromboangitis obliterans and 80 arteriosclerosis obliterans. The latter group included 20 diabetic subjects.

The original thesis proved to be valid. A gradually progressive, measurable increase in the magnitude of arterial pulsation in the denervated leg has been a constant postoperative finding. The maximum postoperative increase in pulsation frequently was not attained until a year had elapsed and has been maintained in some instances, for as long as ten years.

This presentation includes a brief discussion of the physiology of collateral circulatory development in the presence of chronic ischemia, the clinical observations giving rise to the original premise, the clinical selection of cases, and the presentation and interpretation of recorded osellometric evidence of the spontaneous development and prolonged maintenance of an effective collateral circulation in cases of chronic obliterating arterial disease of sympathectomized legs.

#### 5 OCCURRENCE OF ANTIHEMAGGLUTININS AGAINST NEWCASTLE VIRUS IN HUMAN RESPIRATORY INFECTIONS WITH A POSSIBLE INSTANCE OF VIRUS ISOLATION

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Newcastle disease, a pneumoencephalitis of fowl causes human conjunctivitis, and serologic evidence suggests it may cause infections resembling poliomyelitis or influenza. The causative virus agglutinates chicken cells and anti-hemagglutinins occur in sera of inoculated or infected fowl.

Having isolated this virus from infected chickens we sought evidence of human infection by serologic studies of patients suffering from influenza or virus pneumonia. Sera of thirty-six such patients collected in the autumn

There were no marked changes in blood pressure response noted in any of the patients studied

This and other preliminary data indicate that a cold stimulus, particularly following a meal, materially increases the positive tests for so called "ischemia of heart muscle" This may be of clinical value in detecting coronary insufficiency

It would appear that the cold stimulation of the external skin of the face, especially about the nose, is one of the factors which may contribute toward initiating attacks of angina in patients walking against a cold wind

## 8 EVALUATION OF THE ZINC SULFATE TURBIDITY AND TOTAL LIPID DETERMINATIONS IN LIVER DISEASE

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(WITH THE TECHNICAL ASSISTANCE OF LILA DOHM, B.S., AND  
ELIZABETH ROBINSON, B.S.)

In order to assess the clinical value of the zinc sulfate turbidity and total lipid determinations as described by Kunkel, a series of 871 consecutive "liver profiles" were studied. These new turbidity tests were contrasted with the routine liver "function" tests simultaneously performed. The 871 determinations were made in approximately 400 patients, more than half of whom had clinical evidence suggestive of liver disease. Needle biopsy of the liver was performed in many instances.

The zinc sulfate turbidity test was contrasted with the thymol turbidity test. 120 units was chosen as the upper limit of normal for the zinc sulfate turbidity, and 50 units for the thymol turbidity. The cases were divided in to the following groups: cirrhosis (nutritional, postnecrotic, and portal), hepatitis (infectious and homologous serum), obstructive jaundice, carcinoma (primary and secondary), and miscellaneous. The miscellaneous group included patients with nonhepatic disease as well as a few with granuloma of the liver and fatty infiltration of the liver.

The zinc sulfate turbidity was elevated while the thymol turbidity was normal in 19.4 per cent of the determinations. The thymol turbidity was elevated and the zinc sulfate turbidity normal in 5.6 per cent of the determinations.

When the determinations were divided according to diagnosis, the following significant data appeared:

(a) In cirrhosis the zinc sulfate turbidity was elevated and the thymol turbidity was normal in 16.8 per cent of the tests, whereas the zinc sulfate turbidity was normal and the thymol turbidity elevated in 3.7 per cent. (b) In hepatitis the zinc sulfate was elevated and the thymol turbidity was normal in 8.5 per cent of the tests, whereas the zinc sulfate turbidity was normal and the thymol turbidity elevated in only 0.7 per cent. (c) In obstructive jaundice and carcinoma of the liver the zinc sulfate turbidity and thymol turbidity tests did not show the significant differences seen in cirrhosis and hepatitis.

It was felt that the zinc sulfate turbidity test was particularly useful in cases of cirrhosis of the liver.

The values obtained for total lipids using the method of Kunkel were contrasted with the values obtained for cholesterol using the method of Bloor. A total of 167 determinations were so compared. It was seen that the values of

the total lipid and cholesterol roughly paralleled one another. The ratio of total lipid to cholesterol was determined and it was found to be  $3.76 \pm 0.7$ . In 17.9 per cent of the determinations this ratio was exceeded. Most of these were instances of hepatitis and/or cirrhosis. The significance of these ratios is to be discussed.

It was felt that these new turbidity tests provide useful information and deserve inclusion in routine liver function studies.

## 9 AN ANTIDIURETIC SUBSTANCE IN THE URINE OF PATIENTS WITH CARDIAC FAILURE

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Recently, much doubt has been cast upon the conventional explanations of the mechanisms involved during cardiac decompensation. Because of the controversial state of this subject at present, it was felt that examination of other factors involved in water balance would be worth while. Therefore patients with congestive heart failure were studied for the presence of increased amounts of antidiuretic material excreted in the urine.

Patients with definite heart failure were selected for study. None with associated kidney disease or cirrhosis were used. Urines from normal subjects and patients with mental diseases were used as controls. Urines were acidified to a pH of 5.5 to 6.5 with 3 per cent acetic acid, evaporated at room temperature beneath a fan, and then dialyzed in a cellophane sac for six to twenty-four hours. Urines were concentrated so that a fifteen minute output sample would equal 2 to 4 cc. and this amount was used as the test dose. Assay was performed on trained normal female dogs in which water diuresis had been produced by giving 35 cc. per kilogram body weight by stomach tube. Urine samples were collected through an indwelling catheter. A decrease in urine output following intravenous injection of the test material was used as a measure of antidiuretic activity and this was compared with the change in urine output following the injection of known amounts of Pitressin.

Ten patients were studied. Of these, three had hypertensive cardiovascular disease, three had arteriosclerotic heart disease, three had rheumatic heart disease, and two had cor pulmonale. Nine of these were found to have amounts of antidiuretic substance equivalent to 0.5 to 1.0 mμ of Pitressin in a fifteen minute urine sample. None of the controls showed any antidiuretic activity in their urine samples. Investigations now in progress indicate that this antidiuretic material is not the same as commercially prepared Pitressin.

## 10 VICARIOUS EXCRETION BY MEANS OF PERGASTRIC INTESTINAL PERFUSION

LIONEL BERNSTEIN, M.D. (By Invitation), PHILIP B. O'NEILL, M.D. (By Invitation), ARTHUR BERNSTEIN, M.D. (By Invitation), AND WILLIAM S. HOFFMAN, M.D., CHICAGO, ILL.

Pergastric intestinal perfusion was carried out in nine preterminal uremic patients as a means of vicarious excretion. The subjects were placed on a Bradford frame with head end elevated. The perfusing fluid was introduced into the stomach and collected from a rectal tube after transit through the entire intestine. To prevent overdistention of the stomach and vomiting suction was

applied at the cardia through a second lumen of the gastric tube. Such a scheme avoided many of the disadvantages of other artificial means of vicarious nitrogenous excretion. It required no elaborate equipment, no surgical procedure, no preparation of sterile solutions, and avoided the danger of thrombosis or peritonitis. It could be instituted with a minimum of delay.

A significant fall in the serum nonprotein nitrogen concentration was recognizable after six to eight hours of perfusion. The nonprotein nitrogen level of the intestinal perfusate was directly proportional to the serum nonprotein nitrogen level and inversely to the rate of flow. The mean intestinal nitrogenous excretion was equivalent to a urea clearance of 13.6 cc per minute (range, 6.9 to 21.0 cc). At the optimal rate of flow of 2 liters per hour, the clearance was of the order of 21.0 cc per minute. In contrast, gastric perfusion alone was only about one sixth as effective.

Blood levels of creatinine, phosphate, and phenols were not appreciably lowered. Alteration of serum sodium, chloride, and bicarbonate levels generally was toward normal.

Serum potassium levels dropped considerably in all instances, though less so when the potassium concentration of the perfusate was made 40 mg per 100 cubic centimeters. In prolonged perfusions, the serum potassium might fall to the dangerously low level of 12 mg per 100 cubic centimeters. The high concentration of potassium in the intestinal perfusate implied the nonosmotic secretion of potassium in the intestinal juice. The total negative balance of potassium was as much as five times as great as the total extracellular content of potassium. The lost potassium was therefore largely intracellular. This made the extent of the fall in serum potassium concentration unpredictable. In perfusions of more than six to eight hours, the potassium loss might be dangerous. Thus is the limiting factor in the use of this method, which otherwise appeared to be of distinct potential value in prolonging life.

## 11. EXPERIENCE WITH HEPARIN-PROTAMINE TITRATION

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Allen and co-workers first noted that in certain hemorrhagic states a heparinemia-like condition exists which will respond to the parenteral administration of protamine or toluidine blue. They devised a heparin-protamine titration technique to detect this state. Using a simplification of this titration, suitable for clinical laboratory analysis, ninety-one tests were performed using forty-eight patients with various hematologic diseases and twenty-three normal subjects. One cubic centimeter lots of blood rendered incoagulable with 90 gamma of heparin are titrated against selected 0.02 cc increments of a standard protamine solution until an end point of coagulation at room temperature in one hour is achieved. Normal end points range from 0.12 to 0.18 cc of solution, depending on potency of successive batches. Tests were read as -, 0, +, ++, or +++ according to the relationship in 0.02 cc increments of the patient to the control value. Of twenty-three tests run on normal subjects, three were -, eleven were 0, and nine were  $\pm$ . Thus a  $\pm$  value is of no significance. Six patients with thrombocytopenic purpura, four with acute leucemia, seven with chronic leucemia, two with aplastic anemia, nine with pernicious anemia, four with polycythemia, three with miscellaneous anemias, five with prolonged roentgen therapy, six with obscure bleeding problems, and one each with multiple myeloma, Hodgkin's disease, and myeloid metaplasia of the spleen were tested. Of these, only three of the four acute leucemias and two of the seven



chronic leucemias showed a significant increase. Interestingly, three of the six obscure bleeding problems showed a - reaction possibly indicating a compensatory increased tolerance for heparin.

## 12 DISTRIBUTION OF BLOOD TYPES IN THE LEUCEMIAS

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AND HENRY G. PONCHER, M.D. CHICAGO, ILL.

Of five hundred thirty two patients with leukemia studied most of whom have been observed over the past twelve years Landsteiner blood groups were recorded in one hundred thirty seven and Rh types in thirty. Four of those with known Landsteiner types were Negro all the others were white. Of those typed, 42 per cent suffered from acute leukemia 45 per cent from chronic 5 per cent from monocytic and 7 per cent from other types of leukemia. The larger group studied consisted of 31 per cent acute 52 per cent chronic, 6 per cent monocytic and 11 per cent miscellaneous varieties. Thus blood groups were recorded in a significantly greater percentage of acute conditions than chronic. The distribution of blood types among the one hundred thirty seven patients was O 39 per cent A 41 per cent B 13 per cent and AB 7 per cent. The distribution of blood types as recorded in over four thousand five hundred recent routine blood bank tests is O 44.1 per cent A 38.6 per cent B 13.2 per cent and AB 4.2 per cent. If the figures from the cases of leukemia are compared by chi square with those of the larger hospital group the probability of differences being due solely to random selection is 0.55. Thus there is no significant difference of distribution between the two groups. Similarly there is no significant variability of blood type distribution in the smaller groups of chronic myelogenous leukemia chronic lymphatic leukemia, and the pooled cases of acute leukemia.

Of the thirty patients Rh typed 77 per cent were positive, 23 per cent negative. Comparison by chi square with the published figures of 85 per cent positive and 15 per cent negative gives a probability of 0.20 revealing no significant difference between the leukemia and the general groups.

## 13 OCCURRENCE OF HEMOPHILIA IN FEMALES

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CHAPEL HILL, N. C.

Hemophilia is considered ordinarily as a disease of the male sex only inherited as a sex linked recessive characteristic through the X chromosome. Of the possible types of crosses in human hemophilia only two, conductor female  $\times$  normal male ( $Hh \times HY$ ) and normal female  $\times$  hemophilic male ( $HH \times hY$ ), are well established. Rare instances are reported of matings between conductor females and hemophilic males. In this cross  $Hh \times hY$  half of the female offspring should be of the genotype  $hh$  and would be expected to be bleeders. However, authentic cases of female hemophilia have not been described. As a result it has been postulated that the gene combination  $hh$  is lethal, or that the bleeding tendency does not become manifest in females.

Recently a strain of dogs with an inherited bleeding disease was discovered. On the basis of the  $Hh \times HY$  cross a sex linked type of inheritance was demonstrated. The clotting defect appears to be identical to that in human hemophilia. As a result of a breeding program carried on in our laboratory bleeding males have been reared to maturity. This report deals

with the mating of bleeder males with conductor females. Six litters from such matings have been studied. Nearly half of the males were hemophiliac, while the remainder were normal. Approximately half of the females were hemophiliac, while the rest showed no evidence of the disease. The distribution into bleeders and nonbleeders in these litters was close to the expected ratio of 1:1 for the cross,  $Hh \times hY$ .

The clotting defect in the affected animals was the same, regardless of sex. All showed consistently prolonged clotting times, normal bleeding times, normal prothrombin values, and a slow disappearance of prothrombin from clotting blood in spite of normal platelet values. The clotting defect was corrected in vitro by normal plasma or small amounts of thromboplastin. Further studies indicated that the bleeder females, just like the bleeder males, lack a plasma factor required for platelet utilization.

No sex difference in the bleeding tendency was observed. Hemarthroses and subcutaneous hematomas have occurred frequently. Some animals have had evidence of internal bleeding. Hemorrhagic episodes have been controlled by plasma transfusions.

The occurrence of hemophilia in female dogs suggests that the gene combination  $hh$  in human beings is not lethal. Absence of human cases may be due to lack of opportunity for their occurrence, or to inadequate investigation of possible cases.

#### 14 OBSERVATIONS ON THE EPIDEMIOLOGY OF INFECTIOUS HEPATITIS

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The occurrence of infectious hepatitis was studied in two widely separated areas in Wisconsin. One region, in the north-central section, consists of a city of 10,000 inhabitants and surrounding rural communities. The other, 250 miles away in the southwestern section, is a prosperous farming region. The geology of the areas is different. The chief method of study consisted of interviews with each individual who had had the disease and with his suspicious contacts. Most of the cases were discovered in this way. An attempt was made to ascertain pertinent facts concerning the development of each case and to document the individual and environmental factors. Similar interviews were obtained with 60 families in which hepatitis had not occurred. Sanitary surveys were made by the State Board of Health, and other data were obtained relative to terrain, climatic variations, and disease rates. For the purpose of this study, jaundice was made a mandatory criterion for diagnosis.

In the northern area, 168 cases of infectious hepatitis occurred, beginning in January, 1947. In the south, 31 cases had appeared, the first in December, 1945. The disease appeared in all months of the year, with a significantly greater number in winter. The greatest number of cases occurred in patients between the ages of 10 and 30, the disease rarely appearing in preschool children or persons over 50. There was a clear tendency to distribution by families. In the 115 families concerned, there were 199 cases (1.7 persons per family). Of the 579 persons in these families, 34 per cent developed the recognizable syndrome. Members of other families in the vicinity were not involved, even though all activities were shared, so far as could be determined. The occurrence of the disease seemed to present a pattern by groups. In the southern region it was confined to an area of approximately four square miles.

Various environmental factors were studied. The drinking water from wells was found to be contaminated in 30 per cent of homes where cases occurred and in only 5 per cent of homes which had been free of the disease. Other considerations tend to minimize the importance of this as the source for transmission. The occurrence of the disease in localized areas by groups seemed to be the most important aspect of these observations.

# 15 RATES OF TURNOVER AND BIOLOGIC DECAY OF CHLORIDE AND CHLORIDE SPACE IN DOGS DETERMINED WITH THE LONG LIFE ISOTOPE $\text{Cl}^{36}$

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The long life radiochloride  $\text{Cl}^{36}$  ( $T_{1/2} \approx 2 \times 10^6$  years) made possible the study of the early concentration time course in the blood serum, biologic decay rates and rates of turnover of chloride in six normal dogs observed continuously for eighteen to thirty one days under controlled metabolic conditions. These experiments yielded the following information:

(1) The mean concentration time course of  $\text{Cl}^{36}$  in the serum for the first sixty minutes after intravenous administration can be expressed by the multiple exponential equation

$$\text{CN}_t = 1250e^{-2.079t} + 680e^{-0.308t} + 144e^{-0.0377t} + 674e^{-0.0002t}$$

where

$\text{CN}_t$  = concentration in the serum at any time  $t$   
0 = natural log

(2) All the serial chloride leaves the serum each minute and about one third of the nonserial chloride returns to the serum each minute. Thus in a dog weighing 4.54 kilograms (10 pounds) with a chloride mass of 5.5 Gm. about 2.75 Gm. of chloride pass into and out of the serum each minute.

(3) The mean  $\text{C}_{1/2}$  value for the serum was 2.51 days (range 2.16 to 3.00), the mean  $\text{U}_{1/2}$  3.50 days (range 2.75 to 4.00) and the mean  $\text{E}_{1/2}$  3.40 days (range 2.75 to 3.80).

(4) The mean recovery was 91 per cent of that injected (range 83 to 97)—88 per cent (range, 81 to 90) in the urine and 2.6 per cent (range 0.6 to 5.9) in the feces.

(5) The mean chloride space in "serum equivalents" was 35 per cent of body weight (range 32 to 38) and the mean total body chloride 6.84 Gm. for a dog weighing 4.54 kilograms.

(6) These and other data to be presented indicate that: (a) The chloride space is larger than previously reported. (b) Chloride cannot be employed as a measure of extracellular fluid space. (c) Chloride is present in considerable quantities outside the extracellular fluids. (d) Man would require a daily intake of 43 Gm. NaCl or 15 to 22 lb. of food to have a diet comparable to that of a dog weighing 4.54 kilograms. (e) A diet of 200 mg. sodium for a man weighing 70 kilograms is equivalent to a diet of 13 mg. for a dog weighing 4.54 kilograms. Thus the usual studies of low sodium metabolism in small animals with "hypertension" must be evaluated cautiously.

The importance of these studies in planning tracer experiments with chloride and bromide and in understanding the metabolism and excretion of chloride and bromide edematous states, intoxication due to radiochloride and radiobromide in atomic warfare and other physiologic processes is evident from the data.

16 ORAL ADMINISTRATION OF VITAMIN B<sub>12</sub> IN PERNICIOUS ANEMIA

## II STUDIES ON THE NATURE AND SOURCE OF INTRINSIC FACTOR

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Vitamin B<sub>12</sub>, when administered parenterally to patients having pernicious anemia in relapse, behaves in the same manner as does the active principle in liver. However, when vitamin B<sub>12</sub> is administered orally it acts as does extrinsic factor, as Berk and co-workers, Hall and associates, and others have shown. Since patients with pernicious anemia lack intrinsic factor, orally administered vitamin B<sub>12</sub> is not utilized, in most cases at least, unless the intrinsic factor is given also, either simultaneously or within a period of six hours (Castle and associates). If the quantity of vitamin B<sub>12</sub> is kept constant, various unknowns can be tested for the presence or absence of intrinsic factor. We have utilized this procedure with seventeen patients having pernicious anemia in severe relapse. The following data have been gathered from these studies:

(1) Intrinsic factor is present in fresh, Berkefeld-filtered, pooled human gastric juice. (2) It is also present in extracts of hog stomach and duodenum. (3) It is destroyed by heating to a temperature of 63° C for thirty minutes. (4) It is not destroyed in human gastric juice when the latter is stored at a pH of 1.7 at a temperature of 5° C for three months. (5) Trichloroacetic acid and cold alcohol (95 per cent) precipitates from human gastric juice (prepared for us by Dr. J. L. Bollman) did not contain the intrinsic factor. (6) In vitro mixtures of pooled human gastric juice and vitamin B<sub>12</sub>, after standing at room temperatures for twenty-four hours, were heated to 70° C for sixty minutes. When these were administered orally to a patient with pernicious anemia in relapse, no hematopoietic response was elicited. (7) The minimal amount of human gastric juice required to produce optimal hematopoietic responses when administered daily by mouth with 5 or 10 µg of vitamin B<sub>12</sub> has not been accurately determined, but in our experience the daily administration of 75 cc, plus vitamin B<sub>12</sub>, has given optimal results. (8) Variation in the response to large quantities of vitamin B<sub>12</sub> administered alone by mouth has been noted. One patient failed to show a hematopoietic response to a single oral dose of 1,000 µg of vitamin B<sub>12</sub> concentrate. A second patient exhibited a suboptimal response to a single dose of 75 µg of crystalline material. From this, and similar observations by others, it is possible to theorize that lack of intrinsic factor in patients with pernicious anemia is not absolute.

17 CONGESTIVE HEART FAILURE AND HYPONATREMIA UNTO  
WARD EFFECTS OF MERCURIAL DIURESIS

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Recent literature concerning the treatment of congestive heart failure has emphasized the value of dietary salt restriction and the frequent use of mercurial diuretics. One of the complications incident to the rapid diuresis produced by this type of therapy is the profound change in the electrolyte balance.

Such an instance was encountered in the case of a 50-year old man who became critically ill and comatose as the result of treatment of his severe congestive failure with low salt diet and mercurial diuretics. It was not until three

days subsequent to the appearance of coma, when the plasma chloride concentration was found to be low that the true pathogenesis of his condition was suspected and treatment with sodium chloride solution seemed justified. The patient improved clinically and this improvement paralleled the restoration of electrolyte balance. Since this initial experience eleven other patients presenting a similar therapeutic problem have been treated.

In patients with well established congestive failure with edema the extracellular fluid compartment is expanded. The removal of the excess fluid in the extracellular space may be accomplished by a combination of low salt intake and mercurial diuretics. During the excretion of this fluid however sodium and chloride are removed at a relatively faster rate than water. Consequently the sodium chloride values fall and the fluid becomes hypotonic. In the presence of subnormal plasma sodium and chloride concentration the diuretic response to organic materials is inhibited. Clinically the situation which at this point presents itself is one of a patient with chronic cardiac failure who in spite of vigorous treatment is getting worse rather than better and does not respond to mercurial diuretics. Dizziness drowsiness muscular pains and apathy appear and if correction with adequate amounts of sodium chloride is not made in time confusion, convulsions and death may result.

#### 18 METABOLIC CHANGES INDUCED BY SUBTOTAL ADRENALECTOMY RESULTING IN CURE OF CUSHING'S SYNDROME EFFECTS OF LATER ADMINISTRATION OF ACTH

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A metabolic study was carried out upon a 22 year old woman with typical Cushing's syndrome proved to be due to bilateral adrenocortical hyperplasia. It affords comparative data in the same person under three separate conditions: (1) before subtotal adrenalectomy, (2) after metabolic and physical normalcy had been established by removal of more than 90 per cent of adrenal tissue and (3) during stimulation of the adrenocortical remnant with large amounts of ACTH (100 mg per day Armour Standard). The data include observations upon the metabolism of nitrogen, carbohydrate, uric acid, glutathione, cholesterol and electrolytes as well as daily excretions of 17 ketosteroids and of 11 oxysteroids.

To be emphasized are the following results and conclusions:

(1) Before operation fasting blood glutathione averaged 26 mg per cent (highest value 28 mg per cent) and carbohydrate tolerance was greatly impaired. Intravenous reduced glutathione (GSH) abruptly converted the diabetic tolerance to normal but it quickly reverted to the diabetic type. Four months after operation fasting blood glutathione averaged 43 mg per cent (lowest value 39 mg per cent). Carbohydrate tolerance was then normal and has remained so.

These observations are in accord with our previous reports upon the role of GSH in ACTH induced diabetes in normal human beings. A deficiency of free sulfhydryl (-SH) groups appears to be importantly involved in the pathogenesis of the diabetes observed in Cushing's syndrome.

(2) Excretion of 17 KS and of 11 OS (both elevated threefold preoperatively) became persistently normal after adrenalectomy. ACTH acting upon the adrenal remnant returned these values to their preoperative levels. They became normal again upon cessation of ACTH.

Upon sufficient stimulation and for, at least, a short period of time, a small remnant of adrenal cortex can give rise to the same quantity of steroidal end products as had been produced by two hyperplastic glands during the active stage of Cushing's syndrome. Conversely, the hyperplastic adrenals of active Cushing's syndrome are not receiving maximal functional stimulation from endogenous ACTH.

(3) Despite an ACTH-induced return of excretory corticosteroids to preoperative levels, some of the preoperative metabolic aberrations failed to reappear. This was true also of the preoperative vascular hypertension.

It is likely that some of the clinical and metabolic manifestations of increased cortical activity are a function of time as well as of intensity, that mildly increased cortical activity of sufficient duration eventually will manifest itself. This may be important with respect to therapeutic uses of ACTH.

## 19 A CLINICAL-PATHOLOGICAL SURVEY OF 108 TUBERCULOUS PATIENTS

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Autopsies were performed on 108 patients who had active tuberculosis. The diagnosis of tuberculosis was established by clinical means in 95 (Group A), but the disease was not recognized before death in the other 13 patients (Group B).

*Group A*—Tuberculosis was the primary cause of death in 73 of the cases in which it was diagnosed before death, but in 18 patients other diseases than tuberculosis were the cause of death. In 13 of these 18 the nontuberculous disease causing death was recognized clinically, in the other 5 it was not suspected. There were four other patients in this group in whom it appeared that the tuberculous process and another disease were of equal importance in the patient's demise. Adequate attempts were made in the treatment of recognized nontuberculous diseases in this group, but in those five patients who had clinically uncovered diseases early treatment might have helped. There were the following conditions unrecognized before death in the five patients in whom the clinical diagnosis of active tuberculosis was made: carcinoma of the pancreas, heart failure due to arteriosclerotic heart disease, pulmonary infarction, and two cases of pulmonary atelectasis.

*Group B*—Analysis of the 13 patients who had active tuberculosis discovered only at the time of the postmortem showed that two died primarily of the tuberculous process, one from acutely disseminated miliary tuberculosis and one from tuberculous peritonitis. The other eleven patients had clinically recognizable nontuberculous diseases and were properly though unsuccessfully treated. The clinically unrecognized tuberculosis involved the lungs and respiratory passages in all but one patient in whom primary tuberculosis of the kidney was found at autopsy. The other diseases which caused death in this group were generally so acute, severe, or advanced that treatment of the tuberculous lesion would have been of little avail.

Accuracy of clinical diagnosis involving both the tuberculosis and other associated diseases in the group of 108 patients was 121 (86 per cent) in 141 instances. Active tuberculosis was missed clinically in 13 (12 per cent) of the total group studied. Nontuberculous diseases went undiagnosed pre mortem in 65 per cent (7).

Tuberculosis services need to seek consultation from other branches of medicine and surgery as much as do medical and surgical services need the help of pulmonary disease students. This need should encourage hospitals to provide frequent cross consultations. Close proximity of tuberculous hospital and sanatorium units to other medical and surgical units will aid in this endeavor. Therapeutic advances not only in tuberculosis but in other diseases as well will then be readily available to the patients.

## 20 POLYCYTHEMIA VERA WITH HEPATIC VEIN THROMBOSIS CASE REPORT WITH SERIAL LIVER BIOPSIES AND APPARENT RECOVERY

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A case is presented of a patient who apparently recovered from spontaneous thrombosis of the hepatic vein (Chiari's syndrome). It is believed to be the first case to be reported in which the patient recovered.

*Case Report*—A 39 year old shipyard machinist came to the Ochsner Clinic on Aug 30, 1948 because of weakness, failure to gain weight, and a feeling of fullness in the abdomen of ten months' duration. Three weeks before admission edema of the legs and ankles and slowly progressive enlargement of the abdomen were noted.

Physical examination and laboratory studies revealed the combined features of polycythemia vera and severe hepatic disease. The patient had a florid complexion with dilatation of superficial vessels and engorgement of the retinal veins. Spider angiomas were noted over the shoulders and chest. The abdomen was filled with fluid. The edge of the liver could be felt about 10 cm below the right costal margin and there was considerable enlargement of the left lobe. The spleen was ballotable 6 cm below the left costal margin. Pitting edema was apparent in both ankles and pretibial areas.

There was pronounced elevation of the red blood cell count, hemoglobin, hematocrit, white blood cell count and platelets. The serum bilirubin value was elevated to a total of 3 mg per cent. Bromsulfalein test showed 46 per cent retention in forty five minutes. Urine urobilinogen excretion was increased and prothrombin time was 27.8 per cent of normal.

The patient was hospitalized on September 6 for paracentesis and repeat phlebotomies, and 7 mc of radioactive phosphorus were given intravenously. Initial liver biopsy revealed considerable engorgement of the sinusoids in the central area of the liver with pressure atrophy and beginning fibrous replacement. A small branch of the hepatic vein was seen in one section with a recanalizing thrombus occupying the greater part of its lumen. Despite repeated low prothrombin times Dicumarol therapy was instituted. Because of recurrent ascites and hemorrhage with paracentesis a peritoneal button was inserted on October 23. With institution of a low sodium diet and repeated administration of mercurial diuretics the patient improved steadily. The liver and spleen showed progressive decrease in size and on discharge, Dec 2, 1948, the patient was free of edema and bromsulfalein retention was reduced to 13 per cent in forty five minutes and total serum bilirubin to 1.16 mg per cent.

During the next few months the patient required hospitalization once for slight bleeding due to Dicumarol poisoning and once for an intercurrent acute infection. Since that time he has continued to improve, has gained weight,

and is now working full time without difficulty. Two subsequent liver biopsies have shown progressive improvement.  $P^{32}$  has been given once since discharge.

The only usual sign of hepatic vein thrombosis which this patient did not exhibit was the presence of pain in the right upper abdominal quadrant. His recovery is believed to be related to the establishment of unequivocal diagnosis by means of liver biopsy and prompt institution of therapy for the polycythemia, with additional control of the ascites and clotting tendencies of the blood during the period of readjustment.

## 21 RENIN SENSITIVITY AND HYPERTENSINOGEN LEVELS IN ADRENALECTOMIZED DOGS

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Studies undertaken in an attempt to clarify the relationship of the adrenal gland to renin sensitivity and hypertensinogen levels revealed a definite vascular responsiveness to renin and the presence of hypertensinogen in adrenalectomized dogs in both the immediate postoperative period and after frank adrenal insufficiency had developed.

In four animals, after determination of blood pressure responses to 1 unit of angiotonin and 1 mg of renin, bilateral adrenalectomy was done in two stages. Four days following the second stage, when definite hypotension and pronounced increase in the blood urea nitrogen indicated frank adrenal insufficiency, the renin and angiotonin responses were determined again in the anesthetized animals. Hypertensinogen assays were made on control and experimental animals in these instances. In three of the dogs the intravenous injection of 1 mg produced essentially the same rise after as before adrenalectomy, whereas in one dog the response was somewhat less but was proportionately as great when calculated as a percentage of the basal blood pressure. In the hypertensinogen assays the amount of this substance was normal in two and questionably reduced in a third.

In four other dogs anesthetized with pentobarbital sodium four days following the second stage of adrenalectomy, when advanced adrenal insufficiency had developed, injection of 1 mg of renin produced a rise in blood pressure in three instances equal to that produced by 1 to 2 units of angiotonin and in the fourth a smaller rise. In one of these animals 50 mg of desoxy corticosterone glucoside did not increase the vascular response to injected renin. In two dogs in which the second stage adrenalectomy was done immediately prior to assay there was no essential change in the renin response before or after second stage adrenalectomy. In these dogs tachyphylaxis to renin was as difficult to produce after adrenalectomy as it was before.

In one dog studied immediately after bilateral adrenalectomy, the blood pressure subsequently dropped to 10 mm of mercury and little renin response could be elicited whereas the angiotonin response was somewhat higher though still considerably reduced. In one normal dog, rendered unresponsive to renin by repeated injections, 50 mg of desoxycorticosterone glucoside did not materially hasten recovery.



## 22 FURTHER STUDIES ON EFFECT OF DESOXYCORTICOSTERONE ACETATE IN EXPERIMENTAL HYPERTENSION

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Studies of the blood pressure response in hypertensive and normal dogs revealed (1) a usual but not invariable transient pressor effect from intra venous injection of 2 cc of propylene glycol (2) no further effect from the addition of 5 mg of desoxycorticosterone to this material, (3) abolition of this response by a local or general anesthetic and (4) from continuous subcutaneous administration of desoxycorticosterone acetate in sesame oil, a sustained and equal pressor effect which could be abolished by elimination of sodium from the diet and quickly precipitated by addition of large amounts of sodium during administration

In all instances direct blood pressures were taken by femoral arterial puncture and the use of a mercury manometer Hypertension was induced in the dogs either by use of perineal silk wrapping or by application of Goldblatt clamps Several months were allowed to elapse for standardization of pre experimental pressures

In thirty eight determinations on five hypertensive and thirteen normal unanesthetized dogs, no difference in response to the injection of 2 cc of propylene glycol alone and 2 cc of propylene glycol with 5 mg of desoxycorticosterone acetate was noted In most instances a transient 30 mm rise in pressure was produced by the injection of both desoxycorticosterone and propylene glycol and propylene glycol alone This was believed to be the result of phlebalgia since it was abolished by anesthesia in the dogs

In experiments conducted over a long period four hypertensive and two control dogs were given daily injections of desoxycorticosterone acetate in sesame oil in doses of 0.15 to 1.28 mg per kilogram of body weight In nine of ten experimental periods of six weeks each sustained blood pressure rises of 25 to 50 mm of mercury were noted These were sustained at first but gradually tended to return toward normal and in repeated periods in the same dog tended to be of shorter duration and less magnitude In such instances however, increasing the dose of desoxycorticosterone could again augment blood pressure response These responses which were of similar magnitude in hypertensive and control animals, were preventable by the use of low sodium intake and precipitable by the addition of sodium chloride to the diet in both hypertensive and control animals In one of four trial periods in hypertensive animals forced feedings of sodium chloride alone produced a similar rise in arterial pressure

## 23 AN EFFECTIVE METHOD FOR THE PREVENTION OF RHEUMATIC FEVER AFTER THE DEVELOPMENT OF A STREPTOCOCCAL INFECTION

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From January through June, 1949, at Fort Francis E Warren, over 1,650 consecutive cases of exudative pharyngitis and tonsillitis were observed. These cases were divided into control and treated groups of approximately the same size according to An Force serial number. Treatment consisted of the parenteral administration of crystalline procaine penicillin G (suspended in peanut oil containing 2 per cent aluminum monostearate) in doses of 300,000 units on admission and again in seventy-two hours. Later in the study the dosage schedule was changed to 300,000 units on admission, 300,000 units at forty-eight hours, and 600,000 units at ninety-six hours. Studies for the detection of rheumatic fever were performed between the third and fourth week following the initial infection. Throat cultures and blood specimens were obtained from all patients on admission and at the time of re-examination.

That these infections were almost exclusively streptococcal in origin was established by the presence of group A streptococci in approximately 80 per cent of all the original throat cultures and a diagnostic increase of the anti streptolysin "O" titer in the convalescent blood of about 80 per cent of the control group.

In the entire group, twenty-seven patients developed symptoms suggestive of acute rheumatic fever during a period of from ten to thirty-five days following the initial streptococcal infection. Of these, a definite diagnosis of acute rheumatic fever was made in nineteen patients, seventeen of whom were in the control group and two in the treated group. A diagnosis of possible acute rheumatic fever was made in eight patients, six of whom were in the control group and two in the treated group.

It is concluded that the adequate early treatment with penicillin of streptococcal infections will prevent rheumatic fever in the majority of cases.

## 24 A COMPARISON OF TUBERCULIN AND ARTHUS TYPES OF HYPERSENSITIVITY, IN VIVO OBSERVATION IN THE RABBIT EAR CHAMBER

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(INTRODUCED BY ROBERT G BLOCH, M D)

Using the rabbit ear chamber technique it has been possible to compare in vivo the difference between tuberculin and Arthus types of hypersensitivity. The rabbit ear chamber provides a thin layer of living vascularized tissue 40 to 50  $\mu$  thick which can be inoculated directly. Microscopic observations can be made at frequent intervals under the highest magnification, and the dynamics of tissue change can be watched and recorded with Kodachrome motion pictures.

The tuberculin reaction was studied in five chambers inoculated directly with old tuberculin. One animal was sensitized with bovine tubercle bacilli, strain Ravenel RV, and the others with BCG. Dilatation of blood vessels occurred during the first thirty minutes after inoculation and persisted for at least seventy two hours. Generalized sticking of leucocytes to vascular endothelium developed during the first thirty minutes, persisted for forty eight to seventy two hours, but decreased in extent after thirty six hours. Diapedesis of white blood cells resulted in the accumulation of considerable generalized exudate within two to five hours after inoculation. The exudate increased in density during the first thirty six hours. In three experiments localized hemoconcentration due to seepage of plasma through damaged endothelium was followed by stasis and complete thrombosis of small venules. This occurred in the regions of densest exudate twenty two to thirty six hours after inoculation.

The Arthus reaction was studied in six chambers using both bovine albumin fraction V and horse serum as antigens. Immediately following direct inoculation of antigen into the chamber in the sensitized animal platelet thrombi formed in venules and washed away in the general circulation. Within fifteen to thirty minutes there was considerable admixture of white blood cells with platelets in the thrombi which continued to form. Twenty to sixty minutes after inoculation sticking of large clumps of white blood cells and platelets to vascular endothelium was observed in many vessels. Simultaneously other vessels thrombosed completely and plugs of platelets and white blood cells could be seen separating areas of densely packed red blood cells. Dense exudate developed in the regions where sticking was marked, but little or no exudate accumulated around vessels which thrombosed early. The peak of the reaction occurred after eight to ten hours although sticking of white blood cells, vascular dilatation and infrequent formation of new platelet thrombi could be observed after twenty four hours.

Endothelial damage characterized both types of hypersensitivity. In the tuberculin type damage was either primary or initiated by local tissue damage. In the Arthus type it seemed to be initiated by an intravascular reaction.

## 25 ELECTROCARDIOGRAPHIC PATTERNS IN PERSONS OVER 80

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Unipolar multiple chest and limb lead electrocardiograms, in addition to the standard leads, were obtained upon one hundred patients over 80 years of age at the Oak Forest Infirmary and the Illinois Masonic Hospital. The abnormalities are tabulated, the largest groups being: first degree heart block, twenty nine cases; left ventricular hypertrophy, twenty nine cases; bundle branch block, thirteen cases; myocardial infarction, eleven cases. The establishment of the cardiac position using the unipolar leads explained the axis deviation found in the standard leads. In fifty three cases of left axis deviation the heart was in the horizontal or semihorizontal position.

Twenty one electrocardiograms were considered normal using the criteria established by Myers and associates. Of these only fifteen were normal to careful physical examination. Of forty five patients considered normal to physical examination, thirty cases showed abnormal electrocardiograms. Arteriosclerosis and varying degrees of heart failure accounted in the majority for the physical abnormalities encountered.

Our patients being older than the patients of most other published series, together with the use of multiple chest leads ( $V_4R$ ,  $V_3R$ ,  $V_{1-3}$ ), as well as the unipolar limb leads, makes comparison with earlier series difficult. The advantages of the additional leads to electrocardiographic interpretation again become apparent.

## 26 CARDIODYNAMIC AND RENAL STUDIES IN CHRONIC PERICARDITIS WITH EFFUSION, WITH PARTICULAR REFERENCE TO THE MECHANISMS OF FLUID ACCUMULATION

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The cardiodynamic and renal changes in chronic pericarditis with effusion were investigated in the dog with particular reference to mechanisms of fluid retention in relation to congestive heart failure.

Pericarditis was induced with irritative cellophane. Post-mortem morphologic studies revealed chronic nonbacterial pericarditis with effusion, passive hyperemia of the liver, kidneys, and lungs, and anasarca.

The following determinations were done serially on unanesthetized dogs: (1) cardiac output (CO), (2) renal plasma flow (RPF), (3) glomerular filtration rate (GFR), (4) Na clearance, (5) plasma volume, (6) thiocyanate space, (7) hematocrit, (8) plasma proteins, (9) central and peripheral venous pressure (VP), (10) arterial blood pressure, (11) heart rate, (12) intra-pericardial pressure, (13) weight.

As pericardial effusion developed, peripheral and central venous pressure rose. This was the initial change recorded. No dog had an increased plasma volume at this time. In some animals thiocyanate space was normal, in others it was moderately increased. Resting CO, RPF, and GFR were all normal at this time. Na clearances suggested slightly decreased Na excretory rate.

With progression of tamponade, VP rose further. Plasma volume was increased in some animals, at control level in others. Thiocyanate space was elevated. In this late phase, resting CO, GFR, and RPF were at control levels. Arteriovenous  $O_2$  difference was increased. Ability to increase CO in response to excitement and exertion was severely limited. Blood pressure and pulse pressure were moderately depressed.

Prior to exitus circulatory collapse occurred, with hypotension, markedly elevated VP and intra-pericardial pressure, decreased right atrial effective filling pressure. Resting CO, RPF, and GFR were depressed. Na clearance was severely impaired, with prolonged retention of infused saline.

It is concluded from this study that in pericarditis with tamponade (1) Venous pressure rises as increased intra-pericardial pressure jeopardizes right atrial effective filling pressure. Increased venomotor tone appears to be a key factor in the VP rise.

(2) Elevated hydrostatic pressure leads to edema formation. This occurs despite normal resting CO, GFR, RPF, and plasma volume.

(3) It is suggested that with elevated renal venous pressure, increased tubular Na reabsorption brings about Na and  $H_2O$  retention. Plasma volume is thereby maintained despite increased hydrostatic pressure, edema develops without hemoconcentration. Inadequate CO during activity may also contribute to Na retention due to an inordinate fall in RPF and GFR.

(4) Preterminally, a critical level of intrapericardial pressure is reached (10 to 15 cm of H<sub>2</sub>O) beyond which V P cannot rise to maintain right atrial effective filling pressure and C O Circulatory collapse ensues All mechanisms for Na and H<sub>2</sub>O retention are aggravated Exitus soon supervenes

## 27 CLINICAL AND EPIDEMIOLOGIC STUDIES OF MUMPS EMPLOYING THE COMPLEMENT FIXATION TEST

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The complement fixation test has been employed for a clinical and epidemiologic study of mumps Data have been obtained concerning the persistence of antibodies following infection the occurrence of unrecognized infections, the diagnosis of suspected cases of mumps and of "aseptic meningitis" and a comparison of the "S" or soluble antigen and the "V" or virus antigen The results confirm and extend data presented previously from other laboratories

Tests on one hundred fifteen single sera employing infected allantoic fluid ("V" antigen) detected antibody in the sera of fifty of fifty one individuals who gave a definite past history of mumps This positive correlation is notable because the majority of these persons had had mumps ten to thirty years previously There were seventeen individuals whose sera contained no detectable antibody and it seems highly significant that fourteen of them had no history of mumps two had an equivocal history and only one had had mumps Sera from thirty two or 70 per cent of forty six individuals with a negative past history of mumps contained antibody, indicating that inapparent or mild infections are frequent

Diagnostic tests were made with acute phase and convalescent phase sera from thirty four patients with definitive results Twelve sets of sera showed large increases in titer eleven of these were from cases of mumps many with complications, and the twelfth was from a patient in whom mumps was strongly suspected Twenty two sets showed no significant change in titer, all were from patients in whom mumps was considered in the differential diagnosis and thirteen were from patients with "aseptic meningitis"

Tests employing "S" antigen indicated as Henle has reported, that antibody to this antigen usually does not persist as long as antibody to the "V" antigen "S" antigen afforded no decided advantage over "V" antigen for the detection of mumps with paired acute and convalescent phase sera

It is concluded that the complement fixation test is a valuable tool for clinical and epidemiologic studies of mumps In addition, the test has been of practical clinical importance in certain instances, such as in patients exposed during early pregnancy and in professional personnel

## 28 DETERMINATION OF TOTAL BODY SODIUM IN MAN WITH RADIOSODIUM<sup>24</sup>

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Previous methods for determining the sodium content of the human body have been based for the most part, on calculations from chemical analyses of various organs and an assumed ratio of these organs to the total mass of the

body Carcass analysis is obviously more accurate, yet reports of such analyses are available only for the fetus and newborn. Since knowledge of the sodium content of the body should be useful in estimating parenteral fluid requirements, an attempt was made to determine it by the isotope dilution principle.

Intravenously administered radiosodium quickly mixes with intravascular sodium and within two to three hours is in equilibrium (except for cerebrospinal fluid) with extravascular sodium. Equilibration with the sodium of brain and bone takes place more slowly but is believed to be complete in about eighteen hours. When equilibrium is complete, serum specific activity should equal total body specific activity, and from knowledge of the former, total body sodium can be calculated as follows:

$$\frac{\text{Na}^{24} \text{ injected} - \text{Na}^{24} \text{ excreted}}{\text{Serum Na}^{24} / \text{serum Na}^{23}} = \text{Total body Na}^{23}$$

Thirty determinations on twenty-seven healthy young men indicate that total body sodium has an average value of 41 meq per kilogram. In a series of seventeen children the average values are 43 meq per kilogram for the older children, 49 meq per kilogram for large infants, and 82 meq per kilogram for small infants. Comparison of these results with those of actual chemical analyses from the literature will be made.

## 29 COMPARISON OF THE ELECTROPHORETIC PATTERN OF SERUM AND PLASMA IN LIVER DISEASES WITH SPECIAL REFERENCE TO THE GAMMA GLOBULIN FRACTIONS

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Electrophoretic studies on patients with chronic liver disease revealed marked elevations of the fibrinogen peak and lower than expected levels of gamma globulin. To study this phenomenon, we made a comparative study of sixty-five pairs of plasma and serum from persons having various liver diseases, a miscellaneous disease group and a normal group. Electrophoretic studies were supplemented by chemical protein partition (Wolfson and Cohn), flocculation, and other hepatic tests in all except a few miscellaneous cases.

Chemical analysis did not agree with the marked electrophoretic elevations in fibrinogen, nor were fibrinogen peaks completely obliterated upon electrophoretic analysis of the serum. Total serum gamma globulin was higher than the apparent plasma electrophoretic gamma globulin. The serum gamma globulin appeared electrophoretically as two portions of different mobilities, one migrating in the fibrinogen range. This latter portion accounted for the difference between the serum and plasma gamma globulin and, having a similar mobility to fibrinogen, was buried in that peak in plasma determinations. This gamma<sub>1</sub> fraction added to the plasma gamma globulin gave values similar to those of serum and chemically determined gamma globulin. It also accounted for most of the abnormal electrophoretic fibrinogen elevation and when subtracted from the apparent fibrinogen, chemical fibrinogen values were approached. This gamma<sub>1</sub> fraction is apparently identical to the gamma fraction found by Deutch in normal individuals. In our normal group it formed

approximately 24 per cent of total serum protein and 16.1 per cent of total gamma globulin. In acute hepatitis it was somewhat higher than normal, averaging 39 per cent of total serum protein and 16.7 per cent of total gamma globulin. In obstructive jaundice it was 41 per cent and 24.0 per cent, respectively. In cirrhosis this gamma globulin fraction averaged 7.0 per cent of total serum protein and 22.7 per cent of total gamma globulin. Thus in chronic liver disease in addition to a total gamma globulin increase there is a disproportionate increase in the gamma<sub>2</sub> fraction. Fifteen cases of rheumatoid and one multiple myeloma showed expected total gamma globulin increases but little change in the gamma<sub>2</sub> fraction. The serum and plasma concentrations of albumin, alpha and beta globulins were essentially similar. Although plasma electrophoretic tracings appear to be more characteristic for chronic liver disease than do those of serum, their use in protein partition of liver disease is limited because their inability to separate components of the elevated fibrinogen peak gives false impressions of elevated fibrinogen values and lower than expected globulin values.

### 30 INTRA AORTIC BLOOD PRESSURE DURING SURGICAL RESECTION AND REPAIR OF COARCTATION OF THE AORTA

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(INTRODUCED BY HOWARD B. BURCHELL, M.D.)

A mobile oscillographic camera was used during nine operations for coarctation of the aorta to record continuously the electrocardiogram, heart rate, respiration, and direct blood pressure in the right radial artery. The pressure in the aorta above and below the stricture was recorded by direct puncture simultaneously with these other variables before and after repair of the coarctation.

Compression of the area of the stricture produced no significant change in the pressure in the radial artery or in the aorta, whereas compression of the left subclavian artery in four patients produced an average increase of 18 mm. Hg in the systolic and pulse pressures in the radial artery, and an increase of 17 mm. Hg systolic, and 15 mm. Hg in pulse pressure in the aorta above the stricture. In spite of an increased pressure above the stricture this maneuver in the three cases studied produced an average decrease in the pressure in the aorta below the stricture of 7 mm. Hg systolic and 5 mm. Hg, mean.

In eight patients the average time spent opening the anastomosis was 31 seconds, and during this time the mean pressure in the radial artery decreased an average of 11 mm. Hg.

Repair of the coarctation in six patients was associated with an increase of 34 mm. Hg, systolic, and 22 mm. Hg pulse pressure, in intra aortic pressure below the stricture.

In seven patients the onset of the pulse wave in the aorta distal to the stricture before repair was an average of 0.02 second later than in the right radial artery at the wrist, whereas after resection the onset of the pulse distal to the site of stricture preceded that of the wrist by an average of 0.05 second. This was not significantly different from the value obtained from the aorta above the site of stricture.

Correlation between (1) the pressures at operation and (2) preoperative and postoperative direct observations of radial and femoral arterial pressures was not sufficiently close to be evident in this study.

The dynamic responses of the strain gage manometer systems used were studied by measurement of their response to square wave and variable frequency sine wave pressure variations. The response to equal amplitude pressure variations was within  $\pm 8$  per cent of the true pressure change up to 30 cps. Higher frequencies were recorded with diminished sensitivity. The deflection time was 0.02 second and the damping coefficient was 0.62. These dynamic characteristics are superior to those of undamped manometers with resonant frequencies of 150 cps.

### 31 THE EFFECT OF CHOLESTEROL-FREE DIET ON SERUM CHOLESTEROL OF NORMAL AND THIOURACIL-TREATED DOGS

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Experimental studies in dogs indicate that altered thyroid function is necessary for the production of atherosclerosis by cholesterol feeding. In order to elucidate the importance of dietary cholesterol, the following experiment was devised.

Weekly weight and serum cholesterol determinations were made on two groups of four dogs each. After a control period of ten weeks on a commercial dog food, both groups were placed on a diet composed entirely of vegetable products. After a second control period of eight weeks, blood samples were drawn from the hepatic vein and right auricle by means of the catheter technique. Animals in group 2 were then given 1.0 Gm. thiouracil daily. The dose was increased to 1.5 mg. at twenty-five weeks.

In two dogs of group 2, after serum cholesterol had increased to twice the highest control value, hepatic vein and right auricular blood was analyzed for cholesterol.

In group 1, average serum cholesterol was 210 mg. per cent for one dog and 130 mg. per cent for the remaining three animals. This fell to an average of 176 mg. per cent for Dog 1 and 100 mg. per cent for the others. The fall was steady and progressive, lowest levels occurring toward the end of the period.

Group 2 showed striking increase in serum cholesterol after being given thiouracil for ten days. The serum cholesterol reached an average level of 240 mg. per cent over a period of eleven weeks. This approximately doubled average control values.

Comparison of mixed venous blood from the right auricle and hepatic vein showed no significant difference in cholesterol content before and after thiouracil feeding.

It is concluded that (1) the dog can be maintained in good condition on a cholesterol-free diet, (2) all dogs maintained on such a diet show gradual diminution of serum cholesterol, (3) elevation of serum cholesterol produced by thiouracil is not dependent on exogenous cholesterol, (4) excessive production of cholesterol by the liver was not demonstrated in a limited number of animals, (5) the possibility that shift of cholesterol from various organs to the blood serum occurs is being further investigated.



### 32 THE RENAL CAPACITY OF NORMAL HYPERTENSIVE AND CARDIAC FAILURE PATIENTS TO EXCRETE SODIUM

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The capacity to excrete an intravenous dose of 20 Gm of sodium chloride per 70 kilograms of weight was studied in normal hypertensive, and cardiac failure patients. A salt free diet and distilled water were given one day before and for the two days of the experiment. The sodium content of two hour urine specimens was determined for the first twelve hours and for three additional twelve hour periods for a total of forty eight hours following the injection of sodium chloride. These studies were done on eight normal nine hypertensive and eight heart failure patients. The hypertensive patients were studied only if venous pressure kidney concentration tests PSP tests and blood non protein nitrogen showed no evidence of renal failure.

Marked impairment of the ability of the kidney of each patient with cardiac failure and slightly less impairment of the kidney of each patient with hypertension to excrete sodium was observed in every one of the periods studied.

### 33 PRELIMINARY REPORT OF EXPERIENCES WITH RH HAPTEN

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(INTRODUCED BY MAURICE HARDGROVE M.D.)

Hapten is the term introduced by Landsteiner to designate that portion of the antigen complex which determines its specific reactivity. Separated from the protein portion of the antigenic substance the haptene fraction is incapable of calling forth an antibody response when injected into an animal but retains its ability to react with and bind its specific antibody in vitro. We have attempted to extract the haptene portion of the Rh agglutininogen from Rh positive red blood cells with alcohol and ether using a modification of the procedures described by Carter and Price. This material was assayed in vitro by complement fixation, using the method described by Kolmer for the serodiagnosis of syphilis. The hapten material obtained was injected into patients with demonstrable anti Rh antibodies during pregnancy 100 to 200 mg. of the crude material was injected intramuscularly at weekly intervals.

Sixteen of our patients have terminated their pregnancies. Five received eight or more injections and eleven less than eight injections. Of the five receiving "adequate" therapy two delivered Rh negative babies two had macerated stillborn fetuses at thirty two to thirty four weeks and one delivered an Rh positive infant with demonstrable antibodies coating its red cells but no clinical evidences of erythroblastosis fetalis. Eleven patients were seen too late and received only 2 to 6 injections of hapten. Two of these delivered macerated stillborn fetuses, one aborted at eleven weeks one infant was Rh negative, two survived after multiple transfusions, two survived after replacement transfusions and three showed no clinical or hematologic involvement although they were Rh positive and had positive Coomb's tests.

Definite conclusions cannot be drawn from this material. The anti Rh titers of our patients receiving hapten have tended to remain low. In spite of this, our results have been discouraging. For example, one patient delivered a macerated fetus during the thirty fourth week of pregnancy after receiving seventeen injections of hapten. Her titer had never risen above 8 in albumin.

and was only 2 three days before delivery. Another patient who had only four hapten injections delivered a normal Rh-positive infant even though anti bodies could be demonstrated coating its red cells. One must almost conclude that the hapten therapy was not the determining factor.

### 34 CARDIOVASCULAR LESIONS IN RATS SUBJECTED TO GROUP A BETA HEMOLYTIC STREPTOCOCCAL PULMONARY INFECTIONS

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The effect of repeated pulmonary streptococcal infections in the rat was investigated in an attempt to elucidate the nature of the relationship of group A beta hemolytic streptococcal infections to rheumatic fever.

Pneumonia was induced in albino rats by intrabronchial inoculation of group A beta hemolytic streptococci employing techniques previously described. Animals were subjected to from one to seven infections, two strains of streptococci were used alternately in the animals infected repeatedly. Beginning eighteen hours after inoculation, animals were treated with penicillin in amounts previously determined to be adequate for successful control of the infection.

Infections were produced usually at two-week intervals, animals were sacrificed at the end of given periods for study. The hearts were fixed in Zenker formalin, and microscopic sections were stained with hematoxylin-eosin.

Slight to moderate arteritis and periaarteritis of the coronary and/or myocardial arteries occurred in 30 per cent of the experimental animals, whereas 3 per cent of the normal controls exhibited this change. The lesions observed in the experimental group were more intense than those in the control group.

Myocardial lesions, consisting chiefly of pleomorphic cellular foci, were noted in 52 per cent of the animals repeatedly infected, 38 per cent of the controls had similar but less intense lesions.

The incidence of endocardial infiltration by mononuclear cells was slightly more frequent and more intense in the experimental animals than in the controls. Cellular infiltration within the valve substance itself, however, was noted with greater frequency in the control animals. The cardiac lesions observed did not resemble those of rheumatic fever and none of the animals developed arthritis.

Results of this study have demonstrated a significantly greater incidence of cardiac arterial lesions in rats subjected to repeated pulmonary infections with group A beta hemolytic streptococci than in normal control subjects. Single streptococcal infections were not identified with a higher incidence of cardiovascular changes than were found in normal subjects.

The failure of the rat to develop more striking morphologic manifestations of hypersensitivity is in keeping with previous observation. It is believed, however, that the experimental approach used in this investigation, applied to other animal species, may prove a valuable means of studying the problem of the relation of streptococcal infection to rheumatic fever.

### 35 OBSERVATIONS OF THE CHARACTER OF PLATELETS STUDIED WITH A NEW PHOTOGRAPHIC TECHNIQUE

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In previous reports of Butler, Thomas, and Sanford it was observed that in patients receiving intravenous histamine there was no decrease in the number of platelets, but there did occur a slight but definite decrease in the clotting time.

of platelet free plasma, the higher the histamine concentration the greater the decrease. It was also observed by Sanford Butler and Kennedy that the clotting time of children with hemophilia could be greatly reduced by intravenous histamine.

In order to correlate these observations with the disappearance of platelets and increased coagulation in shock we surmised that there must be a simultaneous increased production and destruction of platelets at these clinical levels. To study this theory we have given intravenous histamine to patients using techniques described for coagulation studies but for absolute accuracy, convenience, and a permanent indisputable record we have made serial photographs every three minutes of platelets in solution for many hours.

Platelets examined in this manner can be studied morphologically with great accuracy and appear to remain constant in number for a long time and after a period of settling are found to be of a uniform size and characteristic spindle shape.

**Conclusions**—(1) Platelets are a formed element of the blood. (2) Platelets in solution for many hours in contact with glass do not change their character. (3) A photographic technique for the accurate study of platelets is described.

### 36 THE DIFFERENTIAL DIAGNOSIS OF HYPERGLYCEMIC STATES BY LABORATORY METHODS

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The hyperglycemic state represents a symptom and not a disease and can be produced by various etiological factors. In twenty three patients in whom a disturbance of carbohydrate metabolism was suspected the glucose tolerance and the insulin glucose tolerance were studied in conjunction with other supplementary laboratory tests. The arterial venous difference in the glucose tolerance test was taken as evidence of peripheral glucose utilization. Since this utilization is influenced by insulin a normal arterial venous difference would imply normal insulin effect. Decreased insulin sensitivity was recognized in the insulin glucose tolerance test. It was taken as evidence of the presence of extrapancreatic factors influencing the carbohydrate metabolism. Using these criteria seven of the twenty three cases were recognized as having a normal carbohydrate metabolism. The sixteen patients suffering definitely from hyperglycemia could be divided into the following groups: 4 cases of true pancreatic deficiency hyperglycemia, 3 cases of hyperglycemia showing pancreatic deficiency and extrapancreatic factors, 4 cases of extrapancreatic hyperglycemia and 5 cases of hyperglycemia with insulin and insulin sensitivity. The latter group may include the nutritional, hepatogenous and mild functional hyperglycemias.

The hypothesis is presented that patients suffering from carbohydrate metabolism disturbance may change from one type to another thereby either aggravating or improving the hyperglycemic state. Some confirmation of the foregoing classification was found in the supplementary laboratory evidence and the clinical data available. Patients in whom extrapancreatic influences were postulated as a factor in the hyperglycemia suffered either from obesity or from other demonstrable functional disorders. The possibility that nonglucose reducing substances may interfere with a correct interpretation of the arterial venous differences is discussed and illustrated with a pertinent case.

### 37 THE EFFECT OF 4-AMINO-PTEROYLGLUTAMIC ACID ON THE URINARY EXCRETION OF 17-KETOSTEROIDS AND CORTICOSTEROIDS IN ACUTE LEUCEMIA

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Dougherty and Higgins, working independently, observed atrophy of the thymus, spleen, and lymphoid tissues and hyperplasia of the adenal cortex after administration of toxic doses of 4-amino-pteroylglutamic acid (Aminopterin) to rats. Adenalectomy largely prevented the involution of lymphoid structures. These findings suggested that Aminopterin exerts its effect, in part at least, through the medium of the adenal cortex and led us to study adenal function by means of determinations of the urinary excretion of corticosteroids and 17-ketosteroids of patients with acute leucemia who were being treated with Aminopterin.

In a control series of eighteen patients who had untreated acute leucemia, the values for urinary excretion of corticosteroids were usually within normal limits for adults (0.3 to 1.0 mg in twenty-four hours), in one patient the value was less than normal and in three patients slightly more than normal. Low or low normal values (0.8 to 5.5 mg in twenty-four hours) were found for 17 ketosteroids.

Six patients having acute leucemia were treated with 0.5 to 1.0 mg of Aminopterin daily, there was no evidence of increased urinary excretion of corticosteroids or 17-ketosteroids. In four of the six patients the output of corticosteroids in urine was depressed significantly, and in two of these four it practically disappeared. In the remaining two of the six patients there was no appreciable change from the original values, despite the administration of a total of 23 and 9 mg, respectively, of Aminopterin.

Depression of the urinary excretion of corticosteroids after administration of Aminopterin may result from one or more of three possible causes: (1) cytotoxic effect of Aminopterin on the adenal cortex with reduced activity of this gland, (2) a toxic effect on the pituitary with failure of its adrenocorticotrophic function, and (3) altered metabolism of cortical hormones.

Administration of 25 mg of pituitary adrenocorticotrophic hormone to one patient who was excreting subnormal amounts of corticosteroids was followed by no change in the number of circulating eosinophils and only a 3 per cent increase in the ratio of uric acid to creatinine, which, according to the experience of Thorn, would indicate impaired adrenocortical function. Nevertheless, in response to the adrenocorticotrophic hormone there was a marked rise in the output of corticosteroids which showed that the adenal cortex was still capable of responding to pituitary adrenocorticotrophic hormone and suggests that Aminopterin may effect a depression of the adrenocorticotrophic function of the pituitary.

### 38 HEMORRHAGIC DIATHESIS ASSOCIATED WITH LOW THROMBOPLASTIC ACTIVITY AND A CIRCULATING ANTICOAGULANT

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An acquired hemorrhagic disease occurring in a woman one year after a normal pregnancy and characterized by a prolonged coagulation time, the presence of a circulating anticoagulant, and an apparent deficiency of thromboplastin

has been studied over a period of six years. The hemogram, platelet count, bleeding time, clot retraction, tourniquet test, prothrombin level, plasma fibrinogen, serum calcium, plasma vitamin C and plasma proteins have all been within normal limits on repeated determinations. The administration of large amounts of both fresh whole blood and plasma has not had any effect on the prolonged coagulation time. Precipitins against plasma fractions I and III have not been demonstrable. There was no change in the clotting time of recalcified plasma following centrifugation at high and low speeds. Titration with protamine sulfato revealed no increase in heparin. The presence of a circulating anti-coagulant was demonstrated by the prolongation of the clotting time of normal bloods when serial amounts of the patient's blood were added. The anticoagulant effect was also demonstrable in the plasma. Heating at 60° C for ten minutes inactivated the anticoagulant effect of the plasma. Standing at room temperature or 4° C for forty-eight hours had little or no effect on the anticoagulant. Thromboplastin assay of the patient's plasma compared with normal plasma and a standard preparation of Maltine thromboplastin revealed low levels of available thromboplastin. Plasma staphylococcus coagulase activator was normal. On two occasions electrophoretic studies revealed the presence of an abnormal fast component of the albumin fraction of the serum. Administration of a course of nitrogen mustard to the patient did not alter the clinical or laboratory findings.

The defect in coagulation in this patient is the result of the presence of an abnormal circulating anticoagulant. The findings do not differentiate whether the mechanism involves (1) antagonism of thromboplastin, (2) antagonism of a precursor of thromboplastin or (3) an antienzyme antagonistic to a platelet enzyme necessary for the conversion of thromboplastin precursor to thromboplastin. The end result however is a deficiency of thromboplastic activity in the blood which produces hemophilic type of bleeding in the patient.

### 39 CONTROL OF HEART RATE WITH AN INTRACARDIAC THERMODE

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(INTRODUCED BY HAROLD FEIL, M.D.)

Classical experiments of McWilliam, Flaek and others have shown that the rhythmicity of the S-A node can be altered by thermal changes in excised and perfused hearts and in the exposed heart. We have devised a special thermode which can be placed in the region of the S-A node by venous (jugular, brachial) catheterization of the intact experimental animal.

The thermode consists essentially of a 4 x 30 mm. copper U-tube attached to the cardiac end of a double lumen catheter. The thermode temperature is regulated by perfusing water of various temperatures (4 to 60° C.) through this closed system. In five Nembutalized intact dogs changes in heart rate and in the form of the electrocardiogram were determined by continuous tracings recorded before, during and after thermal changes. In over 100 experiments it was possible to vary the rate in a manner similar to experiments on exposed hearts.

The most pronounced effects occurred when the thermode was located at the junction of the SVC and right atrium in the region of the S-A node. There was a latent period of two to twenty-five seconds. Changes in rate were considered to be due to alteration of the temperature of the region of the S-A node. Cold perfusion caused the sinus rate to decrease from the control level of 120 to 140 to 90 to 80 per minute. When the S-A node region was cooled excessively, the pacemaker shifted to the A-V node, with a rate varying from 57 to 86 per

minute Hot perfusion increased the sinus rate to a maximum of 200 to 232 per minute at 45 to 55° C The acceleration produced by heating was relatively greater and persisted longer than the slowing produced by cooling

Marked changes in rate occurred without T-wave alteration However, in some experiments, when perfusion was rapid there were T-wave changes which indicated that there had been sufficient temperature change of the blood passing the thermode to alter the rate of repolarization of the subendocardial lamina Thus, in accordance with our previous observations, cold perfusion produced large negative T waves in cavitory leads and tall positive T waves in extra cavitory leads, heating produced opposite changes

The intracardiac thermode may prove valuable experimentally and therapeutically

#### 40 THE EFFECT OF RUTIN ON THE HEMORRHAGIC PHENOMENA OF EXPERIMENTAL MALIGNANT HYPERTENSION IN THE DOG

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A striking feature of the malignant phase of experimental Goldblatt hypertension is the occurrence of diffuse hemorrhages The cause of these hemorrhages is unknown, usually being attributed to degeneration and necrosis of arterioles and capillaries

In view of the contradictory reports concerning the effects of rutin in reducing capillary fragility and hemorrhage in the malignant phase of hypertension in man, the following study was undertaken Acute hypertension with uremia was produced by complete bilateral ligation of the renal arteries in sixteen dogs Rutin was administered subcutaneously, 200 mg per day, as indicated in Table I

TABLE I

NUMBER OF DOGS	RUTIN RECEIVED		HEMORRHAGIC LESIONS
	PREOPERATIVELY	POSTOPERATIVELY	
5	0	0	Severe
2	0	Daily	Severe
4	3 days	Daily	Severe
5	10 days	Daily	None

All the animals developed clinical uremia and hypertension, with death in three to six days The tissues were studied as unknowns

Severe hemorrhages in the gastrointestinal tract, heart, pancreas, urinary bladder, diaphragm, spleen, and adrenals, together with myocardial inflammation and necrosis, were seen in the first three groups By contrast, the animals pretreated with rutin for ten days showed complete absence of cardiomyopathy and of the hemorrhagic changes As a result of the complete obstruction of the main renal arteries, coagulation necrosis of the renal cortex and medulla, with an unexpected sparing of the corticomedullary junction, was noted

These experiments demonstrate that the hemorrhages usually seen in acute experimental malignant hypertension were prevented by pretreatment with rutin for ten days prior to operation This protective action may be due to a stabilizing effect of rutin on the ground substance of the arterioles and of the pericapillary sheath The fact that a shorter period of pretreatment did not provide protection suggests that all our animals were relatively rutin deficient

and that saturation required more than three days of pretreatment. The contradictory reports on the effect of rutin in malignant hypertension in man may also depend upon the relative rutin deficiencies in the patients studied. These experiments call attention to the possibility that the several dietary regimens now being used in the treatment of hypertension may be deficient in rutin and possibly other metabolites.

#### 41 A CLINICAL EVALUATION OF THE BLOOD "SLUDGL" PHENOMENON

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The bulbar conjunctival capillaries of a variety of medical patients were observed with the capillary microscope. Erythrocyte sedimentation rates, erythrocyte counts, and serum protein determinations were made within a few days before or after the observation of the circulation in the capillaries. More than 1,200 observations were made.

In general, the degree of sludging was directly proportional to the sedimentation rate. Sludging was observed in a wide variety of pathologic conditions. Patients with hyperglobulinemia usually had an increased sedimentation rate and well developed sludge. Anemia usually caused an increase in "sludging" and sedimentation whereas erythrocytosis (polycythemia vera or congenital heart disease) always produced an opposite effect.

A discrepancy between the degree of sludging and the sedimentation rate occurred in 17.6 per cent of the 619 cases studied. In 5.5 per cent of the cases there was a minimal degree of sludging with a rapid sedimentation rate. Pneumonia was the most frequent diagnosis in this group. In 12.1 per cent of the cases well developed sludging with a slow sedimentation rate occurred. Heart failure and hepatic encephalosis were the most frequent diagnoses in this group. Well developed sludging in the absence of a rapid sedimentation rate is probably the result of stasis in the capillaries.

These observations indicate that blood sedimentation and blood "sludging" have a common cause which according to Fahræus and others is increased rouleaux aggregation.

#### 42 FURTHER EXPERIENCES IN THE MANAGEMENT OF LOWER NEPHRON NEPHROSIS

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Lower nephron nephrosis was found to occur after a variety of shocklike episodes only a few of which were associated with muscle injury or destruction of blood cells. It was seen in lobar pneumonia, acute pancreatitis, acute enteritis, and diabetic coma. The condition was at times difficult to distinguish from other diseases producing oliguria and hematuria, such as acute glomerulonephritis, and bilateral papillary necrosis. Factors producing rapid dehydration and sodium loss, which ordinarily lead to so-called extrarenal azotemia and which respond to fluid and salt administration, could in more fulminating episodes produce true lower nephron nephrosis. The ultimate clinical diagnosis in the patients who survived rested on the finding of severe oliguria from five to thirteen days unresponsive to management, with spontaneous and progressively

increasing diuresis, and with poor renal function even after subsidence of blood nitrogen levels, which function slowly but ultimately returned to normal.

The original illness which brought on the lower nephron nephrosis was important in the prognosis. If infection or trauma persisted throughout the period of oliguria, tissue breakdown was excessive and blood nitrogen levels rose precipitously. These patients died in uremia and heart failure under conditions in which previously well persons might easily survive. All five deaths in our series of eighteen patients occurred with such complications. All patients whose oliguria was associated with self-limiting or controllable conditions, like that of hemorrhage, transfusion reaction, or controllable infection, survived.

We continue to advocate only conservative management in these cases. In the first six cases, successful resistance to the ravages of uremia was achieved by slow induction of edema of nearly normal electrolyte composition. In the later cases, the same results were accomplished by raising sodium levels to normal by intravenous injections of small quantities of 3 per cent sodium chloride and by oral sodium bicarbonate. Thus edema was kept at a minimum. We continue to allow a soft diet ad libitum and believe the slight increase in blood nonprotein nitrogen concentration and edema thereby produced less harmful than the weakness, thirst, and anxiety produced by water and food deprivation advocated by others. Watchful symptomatic treatment of the emergencies of uremia still remains an important factor in successful management.

#### 43 TURBIDIMETRIC DETERMINATION OF SERUM GAMMA GLOBULINS AS CHECKED BY ELECTROPHORETIC ANALYSIS

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Recently a simple method for turbidimetric estimation of the gamma globulins was described by Hueriga and Popper. It follows closely the procedure of the thymol turbidity test and is based on recording of a precipitate which forms if serum is diluted with an ammonium sulfate-sodium chloride solution, simulating the chemical determination and partition of gamma globulins by Wolfson, Cohn, and others. The results of the turbidimetric method were found identical with this chemical method. The latter is supposed to give comparable results with electrophoretic partition. Nevertheless, the direct results of the turbidimetric readings for gamma globulins were compared with the results of electrophoretic partition in 163 blood specimens of 134 cases which included normal subjects, patients with various liver diseases, multiple myeloma, and miscellaneous disorders. In 58.1 per cent of the cases the difference between the results of the electrophoretic determination in plasma and the turbidimetric in serum was less than 15 per cent, in 71.5 per cent, less than 20 per cent. The results were not comparable at all in only 3.7 per cent of the cases, varying more than 50 per cent. The mean difference between the turbidimetric and electrophoretic method in per cent was  $17.0 \pm 15.3$ , the electrophoretic method giving slightly higher values on the average. In thirty-seven cases in which both determinations were performed in serum, the mean difference was  $13.5 \pm 8.4$  per cent. The differences were not more marked in pathologic conditions than under normal circumstances. This comparison indicates that the simple gamma globulin turbidity test may replace the electrophoretic determination of gamma globulins in clinical use. The results of the gamma globulin turbidity test are not identical with those of the zinc sulfate turbidity test of Kunkel, especially in hepatic disorders, since the latter may appear depressed in intra- and extrahepatic obstructive jaundice. This depression indicated by an abnormally low ratio between zinc sulfate and gamma globulin turbidity may therefore have diagnostic sig-



nificance. The normal gamma globulin turbidity values range between 0.60 and 1.25 Gm per 100 ml of serum. Any elevation above this level is significant. The gamma globulin turbidity values are markedly elevated in liver disorders especially in cirrhosis and viral hepatitis and also in rheumatic diseases, several forms of chronic tuberculosis, collagen diseases, chronic skin diseases and other chronic infections. Comprehension of the exact diagnostic value of elevation of the gamma globulin turbidity in the latter conditions requires additional investigation.

#### 44. AN UNUSUAL CLINICAL PICTURE RESEMBLING PROLONGED SERUM SICKNESS "THOUGHT TO BE CAUSED BY TRICHINOSIS"

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A young Negro laborer previously well except for nonseasonal attacks of asthma since childhood was hospitalized with an illness of one month's duration, characterized by an abrupt onset of fever, migratory arthritis, and severe pain and tenderness in the leg muscles. He had received no serum or drugs prior to this illness. When first seen the muscle pain had largely subsided, but he had persistent fever and arthritis and physical examination revealed, in addition, pronounced enlargement of subcutaneous lymph nodes. There was no rash or demonstrable edema. The clinical picture suggestive of serum sickness was supported by laboratory observations of marked leucocytosis, eosinophilia of 4 to 8 per cent, marked elevation of serum gamma globulin as evidenced by the zinc sulfate turbidity test, and the presence of serum heterophile agglutinins of the Forssman type in rising titer. Lymph node biopsy in the fifth week of illness showed marked hyperplasia and muscle biopsy in the sixth week revealed no trichinae or arteritis. There was a strongly positive immediate reaction to dilute trichina antigen injected intradermally. An electrocardiograph showed changes in T waves and QRS complexes suggestive of myocardial disease. Fever, leucocytosis, and joint pains slowly subsided over a six week period and there was a gradual decrease in the size of the lymph nodes. Pyribenzamine treatment during the stage of acute illness resulted in a slight decrease in fever and a definite improvement in the arthritis pain, and objective joint changes decreasing promptly and recurring explosively on two occasions after withdrawal of the drug.

On re-examination, eight months after the onset of the illness, he was asymptomatic and showed on examination nothing abnormal except for palpable lymph nodes of much smaller size. The leucocyte count was normal, but with 4 to 5 per cent eosinophiles. Heterophile agglutinins had disappeared from the serum. The electrocardiograph now showed a P-R interval of 0.22 second.

Trichinosis is suggested as the probable cause of this illness on the basis of the muscle pain and tenderness at its onset, the strongly positive intradermal test with trichina antigen, the persistent eosinophilia and the electrocardiographic abnormalities. This suggests the possibility that on occasion the clinical picture of trichinosis may be chiefly one of hypersensitivity to the trichina antigen. In this connection the report of Reimann, Price and Herbut on the association of trichinosis with lesions of periaarteritis nodosa is of interest.

#### 45 FLUID AND ELECTROLYTE BALANCE IN THE MANAGEMENT OF ACUTE RENAL INSUFFICIENCY

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Studies of Na, K, Cl, N, and water balance were carried out for periods of four to sixteen days in four patients with severe acute renal insufficiency due to lower nephron nephrosis, studies of sodium and water balance were continued for longer periods in these patients and in six additional ones. Calculations of extracellular-intracellular partition of water, sodium, and potassium were made in the four patients according to the method of Darrow.

Marked fall in plasma sodium was encountered during the oliguric phase as well as during the diuretic phase. The drop during the oliguric phase was due partly to dilution in extracellular fluid and partly to intracellular migration. The drop during the diuretic phase was traceable to the inability of the damaged kidney to retain sodium. Spontaneous resumption of capacity to conserve sodium tended to occur about five to seven days after the onset of diuresis. The importance of following plasma sodium levels and using these as a guide in the treatment will be stressed. Another cardinal feature in the treatment, namely, the daily calculation of fluid balance and utilization of this as an index to fluid intake, will also be brought out.

The plasma potassium did not rise to toxic levels in any of our cases. In one patient who went through ten days of extreme oliguria following circulatory collapse due to diabetic coma, the plasma potassium remained below toxic levels as a result of intracellular uptake of potassium which was released from endogenous protein catabolism. Impairment in capacity to conserve potassium was demonstrated during the diuretic phase and in some cases necessitated administration of supplementary potassium chloride to combat hypokalemia.

#### 46 THE EFFECT OF X-IRRADIATION ON ANTIBODY FORMATION

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Hektoen, in 1915, first demonstrated that total body exposure of experimental animals to ionizing radiation suppressed the usual antibody response to antigens injected shortly before or shortly after irradiation. Hektoen's observations led him to ascribe this suppression to the destructive effect of radiation on the lymphatic tissue.

This communication relates an attempt to study the capacity of the rabbit to form antibodies during a period in which all lymphatic tissue of the body, with the exception of the spleen or appendix, is essentially destroyed.

Young adult rabbits were given 800 r total body x-irradiation (250 kv) twenty-four hours prior to intravenous immunization with sheep cells (1 cc of a 2 per cent suspension). Hemolysin titers were determined on serum obtained before immunization and at seven, fourteen, twenty-one and twenty eight days afterward. Animals thus irradiated and immunized either developed no demonstrable hemolysin titer or developed titers of 1 to 80 or 1 to 40 which were demonstrated on the twenty-first day and the twenty eighth day, respectively, after immunization. In the normal animal hemolysin titers of 1 to 5120 were produced by the fourteenth day after the intravenous administration of the antigen, diminishing to titers of circa 1 to 160 by the twenty eighth day. Rabbits given 800 r total body x-irradiation exclusive of the surgically mobilized, lead pro-

ected spleen or appendix produce antibodies to this antigen in a manner comparable to normal nonirradiated control animals except that the maximum titer of 1 to 5120 is usually reached on the twenty first day rather than on the fourteenth. Surgical mobilization of the spleen or appendix was performed while the animals were under Nembutal anesthesia. In the experiments involving spleen protection, the spleen was drawn through a left upper quadrant incision and placed in a lead box one fourth inch thick with an opening for the pedicle only, during the period of irradiation. In the experiments involving appendix protection, the method was similar except that the appendix was drawn through a right lower quadrant incision and a cylindrical lead shield open at one end was used.

These experiments corroborate Hektoen's original classic findings that antibody formation is suppressed by total body irradiation. In addition it has been demonstrated that if the spleen or appendix of the rabbit is protected by lead shielding during irradiation of the balance of the body the capacity to produce antibodies to an injected particulate antigen in an essentially normal manner is retained, even though lymphatic tissue elsewhere in the body is largely destroyed.

#### 47 RENAL AND EXTRARENAL DISPOSAL OF CHORIONIC GONADOTROPIN IN THE IMMEDIATE POST PARTUM PERIOD

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The fate of chorionic gonadotropin in the human being has not been well established. Not more than 20 per cent of chorionic gonadotropin administered parenterally to men appeared in the urine\*. Similar experiments in nonpregnant women indicated occasionally much larger urinary recoveries leading to the view that relatively little destruction or utilization of the hormone occurred in the body†. The foregoing results however are not necessarily applicable to the fate of the hormone during pregnancy.

We have studied the renal and extrarenal disposal of chorionic gonadotropin in fifteen pregnant women. Starting immediately on placental delivery samples of venous blood were taken at three hour intervals for two or more days. Urine was similarly collected by an indwelling catheter for three or more days. Bioassay of the serum and urine for chorionic gonadotropin was performed by Albert's method‡. The total circulating hormone was estimated at zero time (immediately after the delivery of the placenta) by multiplying the determined concentration of hormone per milliliter of serum by the estimated serum volume. The total amount of hormone appearing in the urine was obtained by addition of the determined values for all samples.

The mean total circulating hormone for the fifteen patients was 36,245 I.U. The mean urinary excretion was 2,105 I.U. Thus a mean of 5.8 per cent of the active circulating hormone was excreted in the urine. 94.2 per cent of it was disposed of extrarenally, presumably by endogenous inactivation or destruction.

This conclusion was further borne out by analysis of the blood and urine curves following placental delivery and by calculations involving the renal clearance of the hormone. In the immediate post partum period the renal clearance was 0.47 ml per minute. At this rate the serum would be entirely cleared

Friedman and Weinstein. *Endocrinology* 1: 489, 1937.

†Bradbury and Brown. *J. Clin. Endocrinol.* 4: 103, 1938. Lloyd and co workers. *J. Clin. Endocrinol.* 9: 68, 1949.

‡J. Clin. Endocrinol. 4: 619, 1948.

of hormone in 106 hours, or cleared to the extinction point of the bio assay in 89 hours. The blood disappearance curves have a mean half-life of 34 hours, or a time for extinction of 93 hours, which is grossly incompatible with the time (89 hours) that would be required for reaching the extinction point if the removal of the hormone from the blood depended entirely on renal excretion. Thus a major route of disposal of the hormone other than renal excretion of the active hormone is present.

## 48 THE MEASUREMENT OF THE PERIPHERAL CIRCULATION

### A QUANTITATIVE STUDY

CARL A. JOHNSON, M.D., CHICAGO, ILL.

To date, the clinical measurement of the peripheral circulation is made by indirect means only. The instrumental methods most commonly used are skin temperature studies, oscillometry, plethysmography, photometry, x-ray visualization of the blood vessels, and the use of isotopes.

It is not the purpose of the present paper to evaluate or discuss the relative merits of the various methods but to present a brief report on the results of the measurement of the peripheral circulation by a new method and to discuss the various components of the peripheral records such as amplitude, crest times, diastolic notch, and the central pulse as related to the peripheral pulse.

Slides will be shown illustrating the quantitative measurement of the pulsatile peripheral circulation in patients with such diseases as hypertensive heart disease, organic occlusive arterial disease, cardiac arrhythmias, and organic valvular disease. A few studies on the effects of smoking in sensitive subjects will be presented.

A special adaptor, for the lantern slide projector, has been made for demonstration purposes. By this means it is possible to project on the screen the circulatory changes from any location in the extremities. During the course of the discussion the circulatory changes from a normal subject will be shown, illustrating the speed and ease with which quantitatively calibrated records can be made.

## 49 COOMBS TITER VARIATIONS IN ACQUIRED HEMOLYTIC ANEMIA

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The erythrocytes from seven of ten patients with acquired hemolytic anemia have shown agglutinability with antihuman serum rabbit serum (direct Coombs test). Of the three instances of hemolytic anemia with negative Coombs tests, one was a severe but transient episode complicating pneumonia and empyema, one had persisted intermittently for six years in an individual with no family history of jaundice and a normal erythrocyte osmotic fragility, and one occurred as the terminal episode in a patient with Hodgkin's disease.

Two patients with Hodgkin's disease, one with carcinomatosis and one with pernicious anemia, experienced hemolytic episodes associated with a positive Coombs test. Agglutination of their erythrocytes occurred only in low dilutions of rabbit serum and decreased or disappeared with the termination of the episode. Because commercially available antiglobulin serum failed to give similar reactions the specificity of the local serum was checked against erythrocytes from sixty patients obtained as random samples from the blood bank. Only one

sample erythrocytes from a female patient hospitalized for transfusion for anemia gave a similar low titer reaction. Erythrocytes from fifteen anemic patients with carcinomatosis, Hodgkin's disease, multiple myeloma, leucemia, Gaucher's disease, hypersplenism, and pernicious anemia all without evidence of hemolysis gave negative Coombs tests.

In contrast to the low titer agglutination observed in patients with symptomatic hemolytic anemia, erythrocytes from three patients with acute idiopathic hemolytic jaundice were agglutinated by high dilutions of antiglobulin serum. The direct Coombs titers persisted at high levels in two patients subjected to splenectomy. The patient without demonstrable serum antibody experienced partial remission; the patient with serum auto and isoagglutinins was not benefited. A third patient without splenomegaly received nitrogen mustard. This did not alter the Coombs titer or the degree of hemolysis and produced a fatal thromboecytopenia.

It is concluded that not all patients with acquired hemolytic anemia have positive Coombs tests and that potent antiglobulin serum is necessary to obtain positive tests in certain other cases. In those cases with low titer agglutination the finding of a positive Coombs test was correlated with the presence of a hemolytic episode. In two patients showing high titer agglutination splenectomy did not lower the agglutination titer of their erythrocytes in antiglobulin serum but did lead to a beneficial decrease in the rate of erythrocyte destruction in one case. Additional study is necessary to determine the effect of detectable serum agglutinins on the rate of hemolysis following splenectomy.

## 50 CLINICAL EXPERIENCE WITH A NEW ANALGESIC AGENT

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(INTRODUCED BY I. W. MADISON, MD)

Clinical trial of a new analgesic, 3-hydroxy-N-methylmorphinan hydrobromide (NU 2206), has been carried out. Of thirty-five patients studied, eleven had advanced malignancy, nine were postoperative, and the remaining fifteen had pain from a variety of causes including renal colic, lupus erythematosus, peripheral vascular disease, rheumatoid arthritis, fracture, myocardial infarction, and congestive heart failure. Experience thus far indicates that the drug has approximately three times the analgesic potency of morphine. In seven patients relief of pain was inferior to that obtained when comparable dosage of other analgesics was used. Nausea and vertigo were encountered though less commonly than with morphine, Dilaudid, or Pantopon. When used over a long period of time, tolerance developed and the dosage had to be increased. Euphoria was noted in only four patients. One patient with advanced peripheral vascular disease received fourteen doses of 3 mg. and twenty-six doses of 5 mg. over a period of sixteen days. Following amputation on the sixteenth day, pain ceased and the drug was stopped without withdrawal symptoms. The most striking results were obtained in the group with advanced carcinoma where in many instances pain could be completely abolished without appreciable sedation or respiratory depression even when as large a dose as 20 mg. was given.

No major toxic effects have thus far been observed.

# 51 THE EFFECTS OF COLD ON MAN SPECULATIONS ON DISEASES OF COLD TEMPERATE CLIMATES, NUTRITION, AND THE PITUITARY-ADRENAL AXIS

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Investigations on healthy young men exposed to the cold have uncovered adaptative changes which might provide clues for understanding the genesis of some diseases most prevalent in cold temperate climates. For example, it has been established for troops in the field that the voluntary caloric consumption is greater the colder the climate. Among men traveling in extreme cold there are, on occasion, alterations in water metabolism and electrolyte balance, creatinuria, retention of ascorbic acid, and other changes reminiscent of those observed in injury and other stress. Recently, metabolic changes were observed in thirty-two heat-acclimatized men abruptly exposed to a cold climate (mean temperature,  $-26^{\circ}$  F) for twelve days. The results were similar in many respects to those in man after injection of adrenocorticotrophic hormone and to the "general adaptation syndrome" of animals during stress. Statistically significant responses during the first day included hypothermia, diuresis, hemoconcentration, eosinophilopenia and lymphopenia, neutrophil leucocytosis with increase in the percentage of young cells, hyperuricemia, hyperphosphatemia, and hyperkalemia, hyponatremia and hypochloremia, transient retention of sodium and chloride, and diminution of ascorbic acid in the blood. Most of these changes reverted to normal after two days of exposure. However, in the last six days of exposure there were an increase in kidney and adrenocortical activity, a test dose of water being excreted rapidly at very low specific gravity, continued eosinophilopenia and lymphopenia, and continued hyperuricemia and hyperphosphatemia.

Mills and others have shown that diabetes, hyperthyroidism, pernicious anemia, Addison's disease, and leucemia are most prevalent in cold temperate climates, especially so in populations exposed to wide seasonal and diurnal variations in temperature. There are other chronic disorders such as multiple sclerosis, the rheumatic diseases, and certain cardiovascular diseases which have a geographic distribution similar to that of the five diseases just mentioned. In man, acute exposure to cold has been said to precipitate pneumonia, nephritis, and attacks of gout.

We accept the concept that climatic or environmental stresses are related to the genesis of certain disease processes in man. We would alter the hypothesis of Mills and others that the development of many diseases is directly related to the metabolic load imposed by stimulating climates to the hypothesis that exposure to cold stimulates the pituitary-adrenal axis. There may then occur pathologic changes, some of which have already been detected in studies of the "general adaptation syndrome."

## 52 PATHOLOGIC AND ELECTROCARDIOGRAPHIC STUDY OF THE AURICLES

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(INTRODUCED BY JOHNSON MCGUIRE, M D)

By current standards, auricular disease is said to exist when the P wave in any of the standard leads exceeds 12 second in duration, or 3 mm in height. The validity of these criteria were studied as follows:

1 Two hundred electrocardiograms on normal subjects ranging from birth to 20 years of age were examined. None of the 200 electrocardiograms exceeded the accepted criteria for auricular damage.

2 One hundred electrocardiograms in which the P wave measurements exceeded the limits mentioned were chosen by random selection. They were classified according to the clinical diagnoses. Abnormal P waves were found in 5 cases of myocardial infarction, 28 cases of hypertensive arteriosclerotic heart disease, 19 cases of rheumatic heart disease, 4 cases of congenital heart disease, 9 cases of cor pulmonale, and in 35 cases comprising a miscellaneous group (beriberi heart disease, thyrotoxic heart disease, etc.). Broad P waves were recorded three times as frequently as tall P waves.

3 Necropsy findings in 122 unselected cases of heart disease were correlated with the P wave in the electrocardiogram. There were 42 cases of myocardial infarction, 28 cases of hypertensive arteriosclerotic heart disease, 25 cases of congenital heart disease, 15 cases of rheumatic heart disease, and 12 cases of cor pulmonale. Pathologic abnormalities of the auricles were found in 58 cases and consisted of dilatation, hypertrophy, intra auricular thromboses, endocardial thickening, or combinations thereof. P wave abnormalities were present in 28, or 49 per cent, of these cases. Where electrocardiographic and pathologic findings co-existed, there was no correlation between the type of P wave abnormality and the particular pathologic change recorded. Electrocardiographic evidence of auricular abnormalities was present in 42 cases of the entire autopsied series. In 14 instances of these 42 cases, or 33 per cent, pathologic confirmation was absent. There were 50 cases in the series of 122 in which neither pathologic nor electrocardiographic evidence of auricular abnormalities was demonstrable.

It was concluded that (1) abnormal P wave changes may occur in the absence of gross pathologic changes in the auricles, (2) normal P waves may exist in the presence of gross pathologic changes in the auricles, (3) no correlation existed between the auricular chamber involved and the particular lead in which P wave abnormalities were found.

## 53 THE PRESENCE OF A PHOSPHATASE IN THE HUMAN AORTIC WALL

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The failure to demonstrate a phosphatase in the human arterial wall has been a subject of significance in the discussion of the pathogenesis of arterial calcification. Since previous investigations appear to have been performed only at alkaline reactions, a study was undertaken covering a wider pH range.

After removal of the adventitia and the external part of the media from human aortas obtained at autopsy, the remaining part of the tissue was ground

finely with water in a Pyrex grinder and the samples were centrifuged. For study of the enzymatic activity portions of the supernatant solution were added to citric acid sodium hydroxide and Veronal sodium carbonate buffers, covering the pH range 3.1 to 10.1. Disodium phenyl phosphate was used as a substrate. Duplicate samples, controls, and blanks were run at each pH level examined. The reaction was stopped by the addition of trichloroacetic acid and the phenol content of the filtrates determined by Fohn and Ciocalteu's method. The enzymatic activity was estimated by subtracting the color of the controls from that of the samples.

The studies showed a significant phosphatase activity with a maximum at pH 5.7 to 5.8. At this optimal pH level a fair proportionality was observed between the amount of supernatant fluid used and the phosphatase activity and between the reaction time and the enzyme activity.

#### 54 THE DIAGNOSTIC VALUE OF HIGH PRECORDIAL LEADS

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High precordial leads taken at the intersections of vertical lines through the  $V_3$ ,  $V_4$ ,  $V_5$ , and  $V_6$  positions with a horizontal line at the junction of the third interspace and sternum have been obtained on approximately 4,000 patients. The findings in these leads were correlated with those in the customary precordial and Goldberger limb leads and with the pathologic findings in 300 cases that came to autopsy.

Cardiac rotation appeared to have a greater influence on the QRS pattern in high precordial leads than in those taken in the usual positions. Clockwise rotation displaced the transitional zone farther to the left in high than in the customary precordial leads and often led to the registration of an RS pattern, typical of the potential variations of the epicardial surface of the right ventricle, in leads high in the axilla as well as in a  $V_L$ . Counterclockwise rotation tended to shift the transitional zone farther to the right in the high than in the routine precordial leads.

High precordial leads were of particular value in the detection of infarcts localized to the basal portion of the anterior or lateral walls of the left ventricle, as demonstrated by pathologically proved cases with diagnostic signs in high precordial but not in the routine precordial or limb leads.

High precordial leads were also of value in the estimation of the basal extent of anterior or lateral apical infarcts, as illustrated by cases of localized anterolateral apical infarction, showing diagnostic patterns in the customary but not in the high precordial leads and extensive anterolateral infarction with diagnostic patterns at both levels. The differentiation from high precordial lead patterns due to right and left ventricular hypertrophy and right and left bundle branch block is illustrated.

#### 55 STUDIES ON THE RENAL TUBULAR TRANSPORT MECHANISM FOR GLUCOSE

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(INTRODUCED BY ROBERT W. KEETON, M.D.)

Recent observations by Conn and co-workers indicate that the administration of adrenocorticotrophic hormone (ACTH) to normal human subjects results in a diabetic state. In addition to hyperglycemia, ACTH is be-



heved to decrease renal tubular resorption of glucose. Glutathione antagonizes this effect of ACTH presumably by reversing the inhibitory action of ACTH on tubular transport mechanisms.

Hyperglycemia has been reported to effect adrenal cortical discharge as reflected by a fall in the lymphocyte count in the rat and also in man. Thus hyperglycemia in itself can be considered as a stress phenomenon.

To obtain quantitative data on the effect of adrenal cortical discharge on the tubular mechanism for glucose it was planned to do  $Tm_g$  determinations in trained dogs before and after the administration of epinephrine. A preliminary experiment resulted in a 70 per cent drop in the  $Tm_g$  and a 77 per cent drop in the eosinophil count following the intravenous infusion of epinephrine (5 gamma per kilogram per minute for one hour). Eight periods fifteen to twenty minutes in duration were conducted in each experiment over a five hour period of glucose infusion. The  $Tm_g$  values for the last two to three periods were within normal limits as reported for the dog (200 to 300 mg per minute). In five of six dogs the initial two to three periods showed depressions of the  $Tm_g$  ranging from 33 to 59 per cent.  $Tm_g$  values as low as 92 mg per minute have been obtained under these conditions despite glucose loads of 15 to 20 times the animal's normal  $Tm_g$  level. In contrast to the gradual rise in  $Tm_g$  as a given experiment progressed the eosinophil count fell continuously to a low value at the completion of each run.

The marked depressions of tubular resorption of glucose seemed to coincide with high environmental temperatures. This is of interest since it is known that heat stress will fire the pituitary-adrenal mechanism. It is postulated that environmental heat stress in combination with the stress of hyperglycemia may have been responsible for the anomalous  $Tm_g$  observations noted in this series of dogs.

## 56 AN INDIRECT QUANTITATIVE METHOD FOR THE ESTIMATION OF HEPARIN ACTIVITY IN VITRO. CLINICAL APPLICATION

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Evidence has been presented which suggests that some hemorrhagic states are due to the presence of excessive amounts of heparin in the blood. In such conditions the antiheparin agents protamine sulfate and toluidine blue may be of great value clinically. A laboratory procedure which will permit the selection of patients for this type of therapy and which can be used to control such therapy is desirable. A method is described which utilizes a modified one stage prothrombin time type test. With this technique it is possible to detect the presence of heparin in amounts as small as 35  $\mu$ g per milliliter of plasma. Under the conditions of this test the ratio of the concentration of protamine sulfate required to neutralize heparin added in vitro or in vivo is 15:1. This ratio appears to be a constant. When the amount of protamine sulfate required to neutralize a certain amount of added heparin is greater than that predicted by this ratio it is concluded that the excess protamine neutralizes endogenous heparin. The utility of the test in the protamine sulfate therapy of certain hemorrhagic states is illustrated by case histories. In practice it has been possible to predict which patients will be benefited by the use of antiheparin agents. It has also been possible to determine the adequacy of the treatment even in the absence of frank bleeding.

## 57 THE EFFECT OF ATROPINE SULFATE AND DIBUTOLINE ON THE NOCTURNAL GASTRIC SECRETION IN MAN

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The effect of atropine and of atropine and adrenaline in oil on the fasting gastric secretion was studied in forty-five patients hospitalized for peptic ulcer. Atropine administered parenterally in dosages of 1.0 to 2.0 mg every 3 to 4 hours exerted a variable effect. A depression in acid secretion occurred in approximately 33 per cent of the studies, the effect was of short duration. Increases in gastric secretion were observed in approximately 26 per cent of the studies. Similar results were obtained with 2.0 mg doses. The action of atropine upon the parietal cell appeared to be related to the state of activity of the cells at the time of administration of the drug. Of the patients with duodenal ulcer in whom a reduction was obtained, the secretory rate in 66 per cent was less than the average hourly rate observed in such patients. However, a depression in gastric secretion was not obtained uniformly in all individuals with low rates of secretion. The reduction in output of acid in individuals with high secretory rates was associated with severe symptoms of atropine toxicity. Atropine also was administered in doses of 1.0 mg simultaneously with 2.0 mg of adrenaline in oil. No additive effect was noted insofar as the volume of gastric secretion was concerned. However, the concentration and output of acid were reduced more frequently by the combination of drugs than with atropine alone.

The effect of a pharmacologically related compound, Dibutolone (Dibutyl urethane of dimethyl-ethyl- $\beta$ -hydroxyethyl ammonium sulfate—Meick) on the nocturnal gastric secretion was studied in fifteen individuals. Dosages of 10 mg given intramuscularly every four hours during the night yielded results similar to those obtained with atropine. Although a decrease in the output of acid was observed more frequently with Dibutolone, the depressant effect, whenever it occurred, likewise was variable and of short duration.

## 58 STUDIES OF BLOOD COAGULABILITY AS INFLUENCED BY DIGITOXIN

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Digitoxin was administered to all subjects used in this study by either the oral or parenteral route. In all instances studies of the coagulation mechanism were performed both before and after digitalization with digitoxin. All subjects used presented no evidence of cardiac decompensation or of thromboembolic disease.

The bleeding time, prothrombin time, and coagulation time (Lee and White) were determined in five patients. Two of these patients received 0.6 mg digitoxin intravenously and three received 12 mg digitoxin intravenously. The aforementioned studies were then determined at intervals of five to ten minutes, one hour, four hours, seven hours, and twenty-four hours. In no instance was there any significant decrease in the bleeding time, coagulation time, or prothrombin time.

Eleven patients were then studied with heparin tolerance estimations before and after receiving a full digitalizing dose (16 mg) of digitoxin administered orally over a twenty-four hour period. No significant change in the

heparin tolerance curves was apparent after digitalization when the test was performed according to the method described by de Takats and associates. In order to make the method more sensitive it was modified by using the Lee and White coagulation time. With this modification six determinations on five patients revealed an increase in heparin tolerance and seven determinations on six patients revealed a decrease in heparin tolerance. It is suggested that the wide variations in these data are due to variations intrinsic in the method rather than to the effect of digitoxin.

The problem was then approached by studying the prothrombin times by a modified Quick one stage technique in twelve subjects. Following digitalization with digitoxin there were no significant alterations in the prothrombin times in any instance. After digitalization the subjects were Dicumarolized in the usual fashion. No inhibition of the effect of Dicumarol by digitoxin as measured by the effect on the prothrombin activity was evident.

Three patients were given Dicumarol so that prothrombin activity was reduced to a range of 10 to 30 per cent. After stabilization of prothrombin activity by the administration of maintenance doses of Dicumarol each of the three patients was digitalized with 16 mg of digitoxin given orally. In none of the three cases did the digitoxin appear to interfere with the effect of Dicumarol as reflected by the prothrombin activity.

Finally the problem was approached by the determination of the coagulation time both by the conventional Lee and White technique and in tubes treated with silicone for the purpose of simulating the endothelial lining of the vascular network. Ten patients were studied by this method for a three day control period. It was noted that there was considerable variation in the coagulation times in the same individual from day to day. This variation was noted in both techniques but was especially evident in the technique employing test tubes treated with silicone. These patients were then digitalized by the oral administration of 16 mg of digitoxin and they were placed on daily maintenance doses of this drug. The coagulation time was studied daily for three days according to the previously mentioned techniques. Again there were no consistent changes in the coagulation time as measured by either technique following the administration of digitoxin.

It is concluded therefore that the methods used in this study have demonstrated that digitoxin administered either orally or intravenously has no effect on the coagulation mechanism of the blood. Therefore if digitalization is indicated this study suggests that there should be no hesitation in carrying out this therapeutic procedure with a view that thromboembolism may result therefrom.

## 59 THE USE OF VIODENUM IN THE TREATMENT OF ULCERATIVE COLITIS

M C F LINDERT M D AND M F KOSZALKA M D MILWAUKEE WIS

(INTRODUCED BY ARMAND J QUICK M D)

A continuous search is being made to find an adequate means of combatting the etiological factors of chronic idiopathic ulcerative colitis. Among the more recent additions to our medical armamentarium is Viodenum. This substance is whole desiccated and defatted duodenum which is believed to contain certain antisecretory and antiproteolytic factors. Thirteen patients with chronic ulcerative colitis in acute exacerbation were treated with Viodenum in addition to the commonly used therapeutic measures. Tablets were administered

only after powder had been given in the initial phase of the treatment and until the acute flareup had subsided. The results are as follows: (1) one patient showed no improvement, (2) exacerbation of the disease was noted in three instances, in one of which the tablets passed through the bowel without disintegration, (3) two patients were forced to discontinue treatment because of intolerance as manifested by nausea, diarrhea, and cramps, (4) definite clinical improvement was observed in six patients, (5) no definite opinion could be reached in two.

## 60 THE EFFECT OF TETRAETHYLAMMONIUM BROMIDE ON THE CARDIAC OUTPUT OF NORMOTENSIVE AND HYPERTENSIVE PATIENTS

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In a previous study we have shown that the fall in arterial blood pressure of normal and hypertensive patients induced by spinal anesthesia is not associated usually with a significant fall in cardiac index. This evidence has been used to support our belief that the cause, or at least the sustaining cause, in essential hypertension is an increased vasomotor tone. In attempting further to elucidate the pathogenesis of essential hypertension, tetraethylammonium was used to lower the blood pressure in patients with essential hypertension, and cardiac output studies were made by the direct Fick method.

Sixty-one cardiac output measurements were made on nine normotensive and eleven hypertensive patients before, during, and after the intravenous injection of tetraethylammonium bromide. The twenty patients were divided into four groups: sedated and unsedated hypertensives, sedated and unsedated normotensives.

In both the sedated and unsedated normotensives tetraethylammonium produced a fall in blood pressure but no significant fall in cardiac index. After recovery from the tetraethylammonium, as shown by the disappearance of tachycardia and the return of the blood pressure to or toward control levels, the cardiac index remained lower than either the control level or the level obtained during the tetraethylammonium effect.

In the unsedated hypertensive group the control cardiac indexes were slightly lower than in the normotensive or sedated hypertensive groups. During the tetraethylammonium effect the cardiac index showed little change and in some cases rose to a level above that of the control value.

In the sedated hypertensive group there was little fall in the cardiac index during the tetraethylammonium period in spite of marked falls in the blood pressure. In no case in the whole series did the cardiac index fall below the normal limit during the tetraethylammonium effect.

In both normotensive and hypertensive patients a sympathicolytic agent such as tetraethylammonium may produce a fall in blood pressure without significant fall in cardiac index.

*(To be concluded in the December issue)*

# AMINO ACID EXCRETION IN DEGENERATIVE DISEASES OF THE NERVOUS SYSTEM

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THE recent investigations of Uzman and Denny Brown<sup>1</sup> and Cooper and co workers<sup>2</sup> have shown excessive urinary amino acid excretion to be a consistent feature of hepatolenticular degeneration even in early and mild states of the disease. Occurrence of this amino aciduria with only minimal evidence of general liver dysfunction suggests that it may represent the result of a specific metabolic defect and that such defect may have a direct relationship to cerebral degeneration. It therefore seemed of interest to study amino acid excretion in a group of patients with other syndromes characterized by chronic neuronal degeneration particularly those of familial type.

A second type of metabolic abnormality in Wilson's disease is indicated by the marked increase in copper content of both brain and liver noted by Haurowitz,<sup>3</sup> Glazebrook<sup>4</sup> and in the well controlled observations of Cumings.<sup>5</sup> Mandelbrote and associates<sup>6</sup> reported high urinary copper output in a single case of Wilson's disease and found that in this condition as in normal persons, the administration of BAL (2,3-dimercaptopropanol) greatly increased copper excretion. BAL might thus provide a tool for the demonstration of a possible relation between these two established biochemical abnormalities. The effect on the amino aciduria of a marked though transient disturbance of copper metabolism as produced by BAL was therefore investigated.

## MATERIAL AND METHODS

Amino acid excretion was studied in representative cases of Huntington's chorea, paralysis agitans, dystonia musculorum deformans, familial spastic paraplegia associated with mental defect and hepatolenticular degeneration. Urine samples were collected over a known time interval usually between two and three hours. In all female subjects and in male subjects uncooperative because of intellectual impairment collection was by indwelling catheter. The total fasting urinary amino acid nitrogen excretion in milligrams per hour was determined by the ninhydrin-carbon dioxide method of Van Slyke, MacFadyen and Hamilton.<sup>7</sup> Analyses for tryptophane were carried out by the *p*-dimethylaminobenzaldehyde method of Bates<sup>8</sup> after mercuric sulfate precipitation as described by Folin and Ciocalteu.<sup>9</sup> Xanthurenic acid was tested for by the color reaction with ferric chloride reported by Lepkovsky and co-workers.<sup>10</sup> Chromatographic analyses for the presence of unusually large amounts of individual amino acids were performed by the method of Dent<sup>11</sup> using the one-dimensional technique with phenol as the solvent. In patients with hepatolenticular

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degeneration, copper was estimated by the diethyldithiocarbamate method of Eden and Green<sup>12</sup> Acid cleaned glass and water redistilled over glass were used throughout in the collections and analyses for copper Blank determinations were performed to correct for minute amounts of copper in the reagents

## RESULTS

With the exception of hepatolenticular degeneration, none of the conditions studied showed a significant elevation in urinary total  $\alpha$  amino acid nitrogen excretion per hour when compared with control values on eight individuals which averaged 4.7 mg per hour, range 3.5 to 6.8 mg per hour (Table I) Excretion of tryptophane and xanthuemic acid did not differ grossly from normal with the relatively crude methods employed Chromatographic studies gave no indication of excessive excretion of any one amino acid The usual finding by this method was two distinct spots with  $R_f$ \* values of about 0.34 and 0.51, identified by superposition with knowns as glycine and alanine As in normal urine, a third faint spot with an  $R_f$  value of about 0.83 also was present occasionally

TABLE I TOTAL URINARY  $\alpha$  AMINO NITROGEN IN DEGENERATIVE DISEASES OF THE CENTRAL NERVOUS SYSTEM

PATIENT	$\alpha$ N (MG PER HR)	PATIENT	$\alpha$ N (MG PER HR)
Huntington's chorea		Dystonia musculorum deformans	
M H	4.4	E S	3.2
	4.0		2.3
			7.9
R G	3.8	W McC	3.6
C W	5.2		
	4.4	Familial spastic paraplegia	
T B	3.5	W McD	3.3
	3.7		3.6
Paralysis agitans		J J	4.1
A H	4.9		4.0
	4.1	Hepatolenticular degeneration	
L F	3.0	A G	19.4
	3.6		16.5
		E R	11.0
		M R	12.0
			13.7

The average value of 14.5 mg  $\alpha$ -amino nitrogen per hour in the three cases of hepatolenticular degeneration confirms the marked increase in urinary total  $\alpha$ -amino nitrogen excretion previously observed in this disease by Uzman and Denny-Brown The absence of such a finding in the other conditions studied suggests that  $\alpha$ -amino nitrogen determination may be of value in the differential diagnosis between Wilson's disease and other diseases which occasionally give similar clinical pictures

The initial urinary copper values of 23.5 and 11.0  $\gamma$  per hour indicate a definitely increased copper excretion in hepatolenticular degeneration when

\* $R_f$  indicates the ratio of the distance along the filter paper moved by the amino acid to the total distance traveled by the solvent.

compared with control figures on four individuals averaging 5.3  $\gamma$  per hour, range 3.0 to 7.9  $\gamma$  per hour. These controls are in good agreement with the more extensive observations of Mandelbrote and co-workers<sup>6</sup> who reported a normal average of 4.85  $\gamma$  per hour range 2.1 to 9.0  $\gamma$  per hour.

Administration of BAL sufficient to increase the copper excretion by more than seven times the pre-BAL figure did not reduce the amino aciduria of hepatolenticular degeneration to the control range (Table II). In the first patient injection of BAL was followed by a reaction characterized by profuse sweating, congestion of the sclera, puffiness of the face, nausea, vomiting of about 100 cc of bright red blood, and almost complete anuria which persisted for about one half hour. Intravenous saline infusion was started as soon as the severity of the reaction became evident. It is possible that the apparent drop in amino acid excretion in the first period immediately after BAL may be in part accounted for by this transient extreme oliguria. Subjective improvement, diminished tremor and increased activity about the ward were noted in this patient following BAL but it is possible that these changes did not exceed the range of natural fluctuation in the disease. This improvement has persisted during the five months of observation since BAL administration. In the second patient the dose of BAL was smaller, reaction was minimal, and urine volume was maintained by constant intravenous saline infusion throughout the experiment. Amino acid output under these conditions showed no significant change. Subjectively and objectively the clinical status of this patient remained unaltered.

TABLE II THE EFFECT OF BAL ON AMINO ACIDURIA IN HEPATOLENTICULAR DEGENERATION

TIME OF COLLECTION PERIOD (MIN)	URINE VOLUME (ML PER HR)	$\alpha$ AMINO N (MG PER HR)	URINE COPPER ( $\gamma$ PER HR.)
Patient A. G. Received BAL 7 mg per Kg intramuscularly			
90 min. before BAL	213	165	23
BAL administered			
0-125 min. after BAL	143	10.6	181
125-190	495	20.8	133
190-357	439	19.0	61
357-482	322	17.9	40
Patient E. R. Received BAL 4 mg per Kg intramuscularly			
125 min. before BAL	85	11.0	11
BAL administered			
0-69 min. after BAL	76.5	11.7	101
69-131	136	14.5	57
131-256	190	12.3	26

It is necessary to emphasize that the small number of observations must limit conclusions from the data here reported. Further, chromatography and the methods used for estimating tryptophane and xanthurenic acid were employed only as screening procedures to detect changes of marked degree. However, they did serve to diminish the likelihood that alteration in excretion of individual amino acids was occurring without change in the total  $\alpha$  amino nitrogen. The method used for determination of total  $\alpha$  amino nitrogen, on the other hand, is one of great precision which appears to exclude increase in total amino acid excretion in Huntington's chorea, paralysis agitans, dystonia musculorum deformans and familial spastic paraplegia and to differentiate these

syndromes from Wilson's disease Increased copper excretion was observed in two cases of hepatolenticular degeneration, but no significant change in total amino acid excretion was found at the time of a marked rise in copper output

#### SUMMARY

1 No evidence of abnormal amino acid excretion was found in cases of Huntington's chorea, paralysis agitans, dystonia musculorum deformans, or familial spastic paraplegia These negative results are contrasted with the consistent amino-aciduria of hepatolenticular degeneration

2 Increased urinary copper excretion was observed in two cases of hepatolenticular degeneration

3 Administration of BAL sufficient to increase the copper output by sevenfold did not significantly reduce the amino-aciduria of hepatolenticular degeneration

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## PARENTERAL NUTRITION

### X OBSERVATIONS ON THE USE OF A FAT EMULSION FOR INTRAVENOUS NUTRITION IN MAN

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**A**N EMULSION of fat consisting of 15 per cent coconut oil, 4.3 per cent dextrose, and a combination of 0.5 per cent soybean phosphatides and 1 per cent polyglycerol esters as stabilizers was reported by this laboratory to have been administered successfully to animals (rats, cats, rabbits, and dogs) and to man.<sup>1</sup> The size of the fat particles of this emulsion was below  $1\ \mu$  in diameter for the most part and none were larger than 3 microns. The preparation of this emulsion has been fully described in earlier papers.<sup>2-4</sup> While this emulsion (referred to as Emulsion 41 in the earlier report<sup>1</sup>) has repeatedly been used with success in man, it was pointed out<sup>1</sup> that it was not completely satisfactory from the clinical viewpoint because after a period of approximately one month it developed pyrogenic properties. The purpose of this paper is to present additional information on the use of Emulsion 41 in man, although neither the cause nor prevention of the slow development of pyrogenic activity is yet clearly understood. However, these studies were all carried out with batches of emulsion less than four weeks of age and hence, within the time period that it may be used clinically without pyrogenic reaction.

#### EXPERIMENTAL

The emulsion used in the present study was of the composition of Emulsion 41 and was prepared in this laboratory. Before clinical use, the sterility of each batch of emulsion was determined and the following routine tests were done: (a) rapid injection (15 ml per kilogram body weight) intravenously into three adult rats daily for seven days and subsequent post mortem examination, (b) rapid injection (15 to 20 ml per minute) intravenously into one adult dog for two successive days in an amount of 5 Gm of fat per kilogram of body weight and (c) rapid injection (10 ml per kilogram body weight) intravenously into adult rabbits for pyrogenic assay. Every batch of emulsion had to pass satisfactorily all of these tests before it was used in the clinic. On the average, an emulsion had been prepared for approximately a week before it was used in man. Plastic disposable infusion sets were employed, and an infusion rate of 4 to 5 ml per minute was used in adults and 1 to 3 ml per minute in children, depending on the weight of the child. Viscosity of the emulsions was such that a No. 26 needle could be employed when necessary.

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terminated. The caloric intake of this patient for the immediate postoperative period is shown in Fig 2. It is seen that during the first three postoperative days, the fat emulsion contributed appreciably to the caloric intake, in fact, during the second and third postoperative days it furnished approximately 75 per cent of the total caloric intake. It was during this critical period that the favorable clinical response was especially noted. Because Emulsion 41 was not available, none was given on the fourth and fifth days. The data in Fig 2 also emphasize quite strikingly the importance of ordinary food as a source of calories.

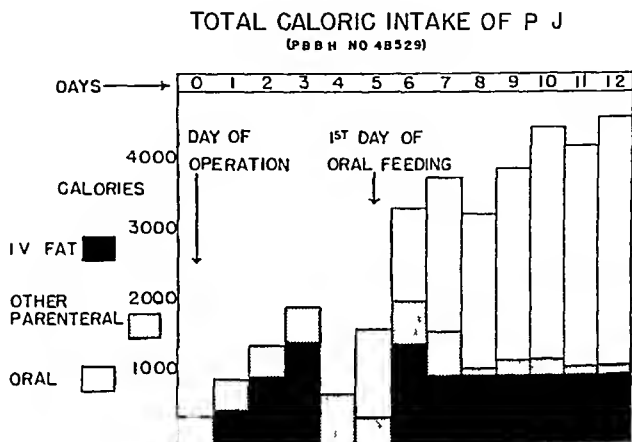


Fig 2

*Patient 9*—This patient was a mentally defective child, 4 years of age, weighing 11 kilograms. Fat emulsion was given on six successive days in amounts varying from 100 to 300 ml, which furnished 30 to 60 per cent of the total caloric requirements. It was observed consistently that infusions of more than 250 ml (3.5 Gm fat per kilogram) at any one time produced an elevation of temperature of 1 to 2° which persisted for one to three hours following the fat infusion.

*Patient 10*—This patient was a 7 week old male infant, weighing 3 kilograms, with postoperative obstruction, who was on continuous gastric suction and complete parenteral feeding. The theoretical caloric requirement for this infant was approximately 300 calories and the usual parenteral fluids available could provide only approximately a third of this requirement. He was given fat emulsion by vein for eleven days, during which time he received on some occasions as much as 70 per cent of his basal caloric requirements from this source. Fig 3 illustrates the caloric intake of this infant during a twelve day period, the last nine days of which fat emulsion was given. Since the two preliminary small infusions of fat were well tolerated, the study presented in Fig 3 was begun. The fat infusions were increased gradually in amount until by the third day they contributed more than one half the total caloric intake (Fig 3). On the fourth day and for the next five days, the theoretical caloric requirement of the infant was more than met, and calories from the emulsified fat furnished approximately two thirds of the total caloric intake. Throughout this period the child was quite ill as a result of an extensive infection. Antibiotic therapy was ineffective and early in the morning of the thirteenth day of this study the child expired. Post mortem studies gave no evidence that the extensive fat infusions, daily for nine days and on two other days shortly before the period represented by Fig 3, had in any way contributed to the death.

*Patient 11*—This patient was a 6½ year old boy, weighing 15 kilograms, with a ruptured appendix, peritonitis, and multiple abscesses in whom complete parenteral nutrition was necessary. He received fat emulsion intravenously for twenty seven days in quantities providing him with 480 to 800 calories per day which provided 50 per cent or more of his

daily caloric requirement. It was observed regularly in this patient that if more than 100 ml of fat emulsion were given at any one time, there was a gradual rise of temperature of 1 to 2 during one to three hours following the infusion. This quantity of emulsion contained 60 Gm of fat which furnished this patient with 4 Gm of fat per kilogram. The infusions of fat in this child were clinically effective in that he presented a problem of complete parenteral feeding and further weight loss was prevented during the period fat infusions were given.

### TOTAL CALORIC INTAKE OF S.E.

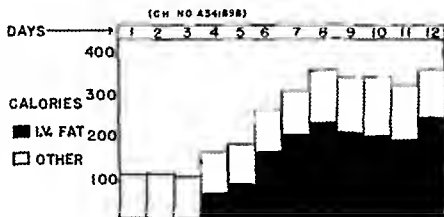


Fig 3

In view of the fact that an increase in temperature of 1 to 2 was consistently obtained in Patient 11 when the amount of fat given exceeded 4 Gm per kilogram body weight it was thought desirable to attempt to determine which component of the emulsion was largely responsible for the temperature rise. The following three different preparations were studied in Patient 11: the usual 15 per cent fat emulsion (Emulsion 41), a 15 per cent emulsion identical to Emulsion 41 except that the costabilizer of polyglycerol esters was reduced from 1 per cent concentration to 0.4 per cent, a fat free emulsion which contained only the two stabilizers in the same concentration as in Emulsion 41 in 5 per cent dextrose. A different one of these three preparations was infused in Patient 11 in the same amount on each of three successive days. For the next three successive days these preparations were given in the same order, but the one without fat was given in twice the amount. The temperature rise occurred only when the amount of fat containing emulsion infused exceeded 4 Gm fat per kilogram of body weight.

That this temperature rise following infusion of a fat emulsion varies considerably among patients is suggested by the observations in Patient 9 in whom an amount exceeding 3.5 Gm fat per kilogram of body weight resulted in a rise in temperature and in Patients 2 and 10 in whom as much as 3 and 6 Gm fat per kilogram respectively did not give rise to an increase in temperature, yet it was the same batch of fat emulsion which was given to Patient 11. The data in Table I show that Patient 5, a 65 year old man, weighing 55 kilograms, with cerebral metastases from a hyaline nephroma, and in whom most of the left frontal lobe had been removed, developed a temperature rise if more than 300 ml of fat emulsion were given at any one time. This amounts to only 0.8 Gm of fat per kilogram of body weight.

Insufficient evidence is available to indicate whether this rise in temperature is due to the amount of fat per se or to pyrogens contained in the fat which, when they reach a certain concentration, give rise to the increase in temperature. At present the latter seems the more likely cause.

## DISCUSSION

The clinical observations presented in this paper indicate that fat emulsions as a source of calories in parenteral nutrition are useful in a wide variety of cases. Emulsion 41 furnished 1,600 calories per liter, and could be infused at the rates commonly employed for the intravenous administration of glucose or saline solutions.

Previous studies in the dog and rat have shown conclusively that such fat emulsions of fine particle size introduced directly into the systemic circulation are utilized for energy purposes as indicated indirectly by total carcass analysis for fat,<sup>4</sup> maintenance of normal weight,<sup>5</sup> conversion of negative to positive nitrogen balance on a low-protein low-calorie ration,<sup>5</sup> and growth of puppies,<sup>6</sup> and directly by radioisotope studies showing that  $C^{14}$  introduced as part of the fat molecule in a fat emulsion is rapidly eliminated in the expired  $CO_2$ .<sup>7</sup> In previous studies with experimental animals and man, it has been shown that fat emulsions such as have been employed in the studies can be given safely and without producing pathologic conditions.<sup>1, 3</sup>

The studies reported in this paper extend many of these observations in man. The observations on Patient 1 confirm the favorable effect of fat emulsions administered intravenously on nitrogen balance. The similar observations on potassium balance further emphasize the necessity of adequate calories for protein synthesis in the body. The very favorable clinical response observed in the patient severely ill with ulcerative colitis (Patient 2) is attributed in part to the calories supplied by the fat emulsion during the first three postoperative days. In Patients 10 and 11, fat emulsions were given for rather long periods, eleven and twenty-seven days respectively, and in generous daily amounts, approximating 6 and 4 Gm fat per kilogram of body weight. Patient 10, who was severely ill from a generalized infection, expired after nine successive days of fat infusion given in such an amount that calories from fat furnished two thirds of the total caloric intake for a period of seven days. However, at autopsy, there was no abnormal accumulation of fat in any of the organs and no evidence that the fat infusions in any way had contributed to the death. During this severe illness, when all nourishment had to be given by vein, weight loss was prevented.

An increase in temperature in Patients 5, 9, and 11 was regularly observed following a single administration of a certain amount of fat. However, the amount of fat necessary to cause this temperature rise varied widely among these three patients—from 0.8 to 4 Gm fat per kilogram of body weight. Patient 2, who received 3 Gm fat per kilogram of body weight, and Patient 10, who received 6 Gm fat per kilogram of body weight, showed no increase in temperature. It is thought that these temperature rises were due to pyrogenic materials in the fat and not to the fat per se.

Of the patients listed in Table I, three died by the time of the preparation of this paper—Patients 5, 8, and 10. Post-mortem examinations were obtained on the latter two and in neither was there any gross or microscopic pathologic condition attributable to the fat emulsions. Patient 5 expired approximately

one month following the last fat infusion Patient 8 three months following the last infusion and Patient 10 eighteen hours following the last infusion of fat

It should be pointed out that the reason more fat emulsion was not given to many of these patients and for longer periods of time was that not enough was available because of limited production and control facilities and the needs of various experimental studies with animals As stated in a previous paper<sup>1</sup> on this subject Emulsion 41 is still not considered completely suitable for clinical use because of the development of pyrogenicity after it is approximately four weeks old

#### SUMMARY

Observations are reported on the intravenous administration of a 15 per cent fat emulsion to eleven patients representing a variety of common illnesses Of these patients eight were adults two were children and one was a 7 week old infant The emulsion furnished 1 600 calories per liter and was given at rates ordinarily used for administering glucose or saline solutions Daily amounts up to 3 Gm fat per kilogram of body weight were given to adults and 6 Gm fat per kilogram of body weight in a 7 week old infant Infusion periods ranged from three to twenty seven consecutive days The emulsion was effective as indicated by favorable clinical response the prevention of weight loss and the maintenance of positive nitrogen and potassium balance Subsequent post mortem examination of three of these patients revealed that the fat emulsions had produced no pathologic changes either gross or microscopic

The authors wish to express appreciation to the Medical and Surgical Services of the Peter Bent Brigham Hospital and the Children's Hospital of Boston for their genuine cooperation in these studies and to The Upjohn Company Kalamazoo Mich which has supplied us generously with various materials used in this research

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# THE ABSORPTION AND DISPOSITION OF ORALLY ADMINISTERED $I^{131}$ -LABELED NEUTRAL FAT IN MAN

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NONE of the available methods for measuring the absorption and utilization of fat in man is greatly informative and, at the same time, accurate and simple to carry out. The direct chemical determinations of serum fat in serial fashion are tedious and time consuming, in the hands of all save the most expert they are subject to considerable inaccuracies. Relatively large samples are required. No information as to the disposition of the fat is available from this method, i.e., what proportions of the lipid which has disappeared from the blood stream are accounted for by degradation and by storage respectively. The vitamin A tolerance test obviates the first two of these objections, but not the third. Nephelometric techniques for fat analyses are now generally conceded to be unreliable.

The problem had been quite satisfactorily solved for animal work by Geyer and co-workers,<sup>1</sup> and Lerner and co-workers,<sup>2</sup> by the use of neutral fat containing radioactive  $C^{14}$ -substituted fatty acids. However, the extremely long half-life of this isotope precludes its use in man. In addition, other isotopes which emit radiation of greater penetrating power are more simple to measure.

Certain of the shortcomings mentioned may be overcome by the use of neutral fat which has been labeled with radioiodine  $I^{131}$  in the unsaturated fatty acids. It is the purpose of this communication to report tracer studies with such radioactive fat on eighteen patients, including ten normal subjects.

## METHODS

Commercial olive oil was iodinated with  $I^{131}$ . \* The following steps were employed. To the carrier free  $I^{131}$  solution (usually 10 mc), 10 mg of sodium iodide were added as carrier. The solution was then placed in a separatory funnel and acidified with 1 ml of 250 per cent concentrated nitric and sulfuric acid mixture. The liberated iodine was extracted with chloroform. Into the chloroform solution a stream of chlorine was then passed until the purple color of iodine just disappeared. The iodine chloride obtained was added to a chloroform solution of 20 Gm of olive oil. This mixture was allowed to stand for twenty-four hours. The chloroform solution then was shaken repeatedly with a solution of sodium carbonate in order to remove any free iodine. The chloroform solution was dried over anhydrous sodium sulfate and the chloroform evaporated off by means of an infrared lamp. The odor, taste, color, and consistency of the oil were unchanged following iodination.

One half to 5 ml of the oil, containing 100 microcuries of  $I^{131}$ , were soaked into bread and eaten, either during or immediately following a normal breakfast. In a few instances the patients were without breakfast all ate a normal mixed diet during the balance of the test period.

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The radioactivity in the thyroid gland was estimated by serial counting at a distance of 35 cm from the neck with a sensitive directionally shielded gamma tube (Sylvania). The background (from extrathyroidal tissues plus cosmic and other extraneous radiation) was calculated by counting over the lower thigh this was subtracted from the thyroid count. The resulting net count was compared with that obtained under similar geometric conditions from a suitable aliquot of the original solution.

One milliliter portions of urine in uniform glass vials were counted with the gamma tube through an orifice in the shield and compared with equal volumes of suitable dilutions of the original solution. Since the bottoms of the vials were in contact with the metal tube surface, excellent reproducibility and sensitivity (272 net counts per second per microcurie) were obtained.

Two tenth milliliter aliquots of serum were pipetted onto flamed, 1 inch copper planchets, each of which was partially covered with a smaller disk of thin absorbent paper. Since the planchets were grease free and were coated with cupric oxide, the liquid was absorbed uniformly into the paper. After slow drying with an infrared lamp, the serum formed an evenly adherent film, the area of which was limited to that of the paper circle. The preparations were permanently mounted by covering with Scotch tape. Standards were prepared by evaporating suitable aliquots of petroleum ether solutions of the original material in the same manner, self absorption was equalized by adding identical amounts of serum. The samples were counted with a thin window Geiger Muller tube.

Two milliliters of serum were diluted precipitated with zinc sulfate and the excess zinc precipitated with alkali by the method of Somogyi. Five milliliter portions of the supernatant (equivalent to 0.5 ml serum) were evaporated onto disks and the radioactivity was compared with similarly prepared standards as described. Since the lipids were precipitated with the proteins, the radioactivity in the watery fraction of the serum represented inorganic iodine which had been removed from the fat. By subtraction of this 'water soluble' portion from the total, a fraction designated as the 'lipid  $I^{131}$ ' was obtained.

In seven patients the sera were separated by means of Bloor's solution and the radioactivity was determined in the lipid and water soluble fractions of this extract. There was in general agreement between the results obtained by this procedure and the former one. However there was usually slightly more radioactivity in the portion resulting from petroleum ether extraction of the dried Bloor's extract than when this lipid fraction was calculated by subtraction. Because of the greater simplicity of the zinc precipitation method it was used exclusively later.

## RESULTS

In ten normal subjects the pattern of absorption and disposition was constant. The concentrations of total  $I^{131}$  in the serum were greatest at three to six hours following the meal and slowly decreased after this time. The peaks in both the 'total  $I^{131}$ ' and 'lipid  $I^{131}$ ' curves occurred simultaneously (Fig 1). These levels of total radioactivity ranged from 0.4 per cent to 0.6 per cent per 100 ml of serum, whereas the highest concentrations of the 'lipid' fractions varied from 0.20 per cent to 0.47 per cent per 100 ml of serum.

The accumulation of radioactivity by the thyroid gland varied from 10 to 25 per cent in twenty four hours (Fig 1). Although appreciable, this was somewhat less than the average collection of a dose of inorganic  $I^{131}$  administered for the purpose of ascertaining thyroid function.<sup>3</sup> Likewise, the excretion in the urine of 15 to 48 per cent of the ingested radioiodine by the normal controls in twenty four hours was distinctly less than if the material administered had been inorganic iodine.

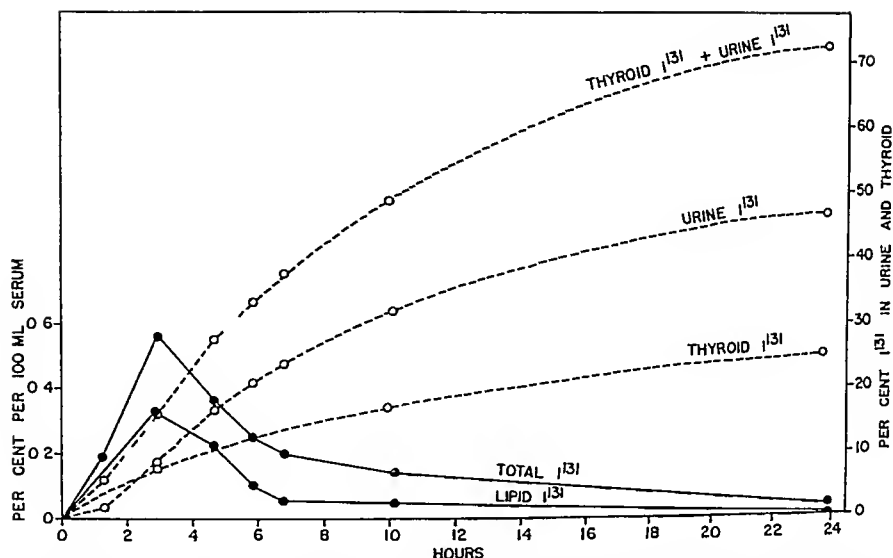


Fig. 1—Curves showing the fate in a normal subject of radioiodine introduced into the body as labeled fat. The time in hours following the ingestion of the iodinated fat is plotted on the abscissa. Along the left ordinate are plotted the concentrations of radioactivity attained on the serum; variations in these values are indicated by the solid lines. The broken lines show the percentage of administered radioactivity which was excreted in the urine and collected by the thyroid (right vertical scale).

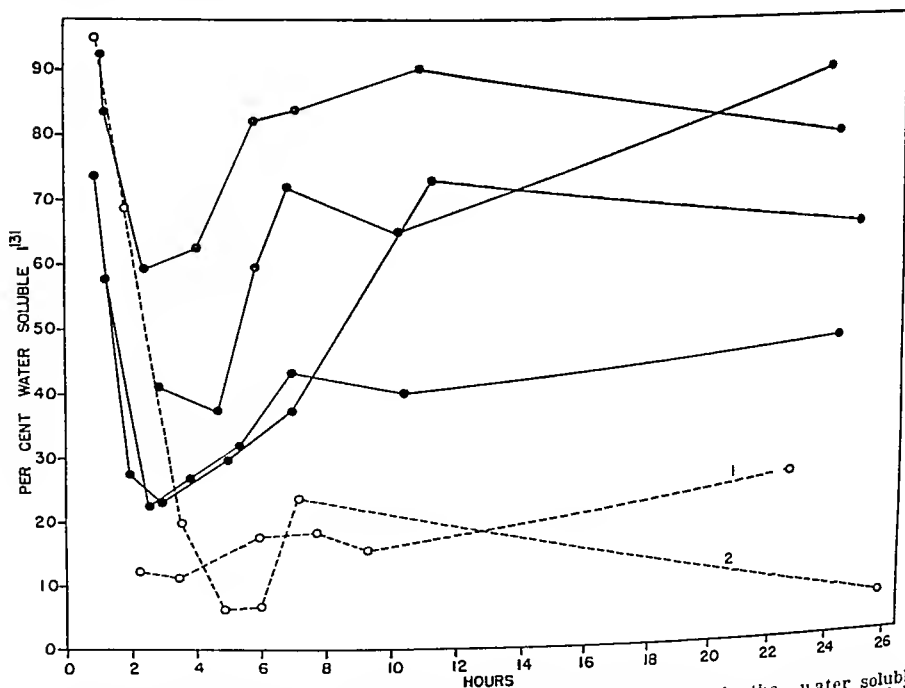


Fig. 2—The variation with time of the fraction of radioactivity in the water-soluble portion of the serum. The sera were fractionated as described in the text; the water-soluble  $I^{131}$  is plotted as per cent of the total radioactivity. The solid lines and circles show the values in four normal subjects while the broken lines and open circles denote a patient with idiopathic hyperlipemia (2) and the nephrotic syndrome (1). In the normal subjects (and in the patient with idiopathic hyperlipemia [2]) during the first hour of the test 60 to 90 per cent of the radioactivity was in the water-soluble fraction. In the normal subjects this sharply decreased during the next one to two hours as absorption of the iodinated olive oil reached a maximum and returned slowly to the high values during the next four to seven hours. In the hyperlipemic patients the percentage of radioactivity in the water-soluble portion of the serum remained low throughout the rest of the test after the first two hours.



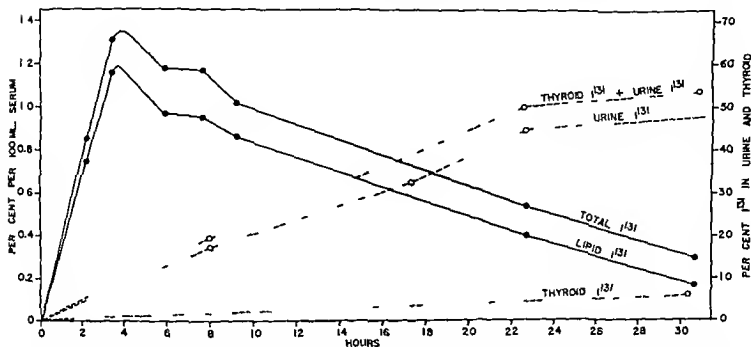


Fig 3—The absorption and disposition of labeled fat in a patient with the nephrotic syndrome. The data are indicated as in Fig 1. It will be seen that the concentrations of radioactivity in the serum principally in the lipid fraction are much greater than normal, the decrease in these high levels is abnormally slow. The excretion of  $I^{131}$  in the urine and its accumulation in the thyroid especially during the early hours of the test are also somewhat slower than normal.

The changes in the ratio of serum moraine to 'lipid' iodine also were uniform (Fig 2). During the first one to two hours from 60 to 90 per cent of the  $I^{131}$  was in the water soluble portion. Later as absorption of the iodized olive oil progressed the size of the moraine fraction decreased sharply then gradually returned to the high values during the ensuing eighteen hours.

In three patients with high serum neutral fat levels (two subjects with 'idiopathic' hyperlipemia and one individual with the nephrotic syndrome) the peak 'lipid  $I^{131}$ ' values were two to five times normal. There was an abnormally slow decrease in the radioactivity during the twenty four hours following these high values. A larger than normal portion of the radioiodine remained in the lipid fraction (Fig 2) and the accumulation of radioactivity in the thyroid and the excretion of the radioiodine in the urine were abnormally slow also (Fig 3). In one patient with hypercholesterolemia xanthomatosis the test was normal. In one case of sprue the highest serum concentrations of total  $I^{131}$  were about one half normal. The urinary excretion and thyroid collection were correspondingly low, but there was a normal relationship between the moraine and 'lipid' fractions in the serum at all times.

### DISCUSSION

It should be emphasized that the procedure under discussion was not a "tolerance test" in the usual meaning of the term. The amount of oil administered was never more than 5 Gm, this served to label the fat being absorbed from the meal eaten with the tracer as well as that already in the blood stream.

Olive oil contains about 80 per cent glycerol trioleate and about 7 per cent trilinoleate. The former of these 18 carbon fatty acids possesses one double bond between the 9th and 10th carbon atoms, while the latter has two such

bonds The saturated compounds tristearate and tripalmitate comprise the bulk of the remainder of the lipids of the oil

As a result of iodination the radioiodine was firmly attached at the double bonds in the unsaturated fatty acid chains, the process used was similar to the familiar method for determination of the "iodine number" of fats In order to be certain that this iodine was organically bound, petroleum ether solutions of the oil in each lot were repeatedly extracted with water, no radioactivity was detectable in the aqueous phase

It may be accepted as proved that the iodine collected by the thyroid gland is in the form of the iodide ion<sup>2</sup> The identity of the iodine excreted in the urine by the subjects in this study has not been determined, although it is entirely likely that it is in the same form The radioactivity in the water soluble portion of the serum and that collected by the thyroid and excreted in the urine represent iodine which has been removed from the fatty acid skeleton during the process of metabolism, these fractions provide an index of the rate of utilization of the iodinated fat

In this study, from 50 to 73 per cent of the administered radioactivity was collected in the thyroid gland and excreted in the urine of normal subjects during the twenty-four hours from the beginning of the test, with approximately one-half these amounts accounted for during the first six hours These figures indicate the extent of degradation of the fatty acid skeleton of ingested neutral fat which normally occurs in men, either as a preliminary to storage in the fat depots or, much more likely, for production of energy The characteristic appearance of an appreciable portion of radioactivity in the water soluble fraction of the serum during the early hours of the test suggests that a small amount of breakdown may have occurred during the process of absorption from the gastrointestinal tract

That a similarly rapid metabolism of intravenously administered neutral fat takes place in the rat has been recently demonstrated by Geyer and co-workers<sup>1</sup> and by Lerner and associates<sup>2</sup> The latter group used a 16 carbon fatty acid (as tripalmitin) with the 6th carbon atom labeled with  $C^{14}$ , as much as 59 per cent of the injected  $C^{14}$  was found in the expired air in twenty-four hours The type of fat administered and the position of the labeled atom were not identical in the experiment of Lerner and co-workers and in the present study However, the curves representing the sum of urinary excretion plus thyroid accumulation of  $I^{131}$  in the normal subjects in this study closely resemble those depicting the cumulative excretion of  $C^{14}O_2$  by the rats in the experiment of Lerner, Charkoft, Entenman, and Dauben

#### SUMMARY

Characteristic curves of absorption and utilization of physiologic amounts of lipid taken by mouth by subjects on a mixed diet were demonstrated by the use of unsaturated fats iodinated with  $I^{131}$  The proportion of the administered radioactive iodine which was collected by the thyroid and excreted in the urine, as well as the magnitude of the water-soluble portion in the serum, indicated

the extent of break down of the labeled fat. Under these circumstances in normal subjects degradation of from 50 to 73 per cent of the orally administered iodinated fat took place within twenty four hours. Subjects with "idiopathic" hyperlipemia and the nephrotic syndrome utilized the labeled lipid much more slowly.

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# THE EFFECT OF SPLENECTOMY ON THE TOXICITY OF $\text{Sr}^{90}$ TO THE HEMATOPOIETIC SYSTEM OF MICE

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## INTRODUCTION

**R**ADIOSTRONTIUM ( $\text{Sr}^{90}$ ), a  $\beta$ -ray emitter with a fifty-five day half life, which is physiologically interchangeable with calcium, localizes largely in bone soon after enteral or parenteral administration<sup>1,6</sup>. The minimum intra peritoneally administered dose required to produce a significant leucopenia in CF-1 female mice<sup>7</sup> was found by Simmons and Jacobson<sup>7</sup> to be about 0.068 microcuries per gram of body weight. However, this leucopenia was relatively transient. Blues and co-workers<sup>8</sup> have reported that bone sarcomas are readily produced in this strain with this dose. Although a severe and persistent leucopenia developed after an intraperitoneal dose of 2.0 microcuries per gram of body weight, no significant anemia occurred. In a preliminary experiment<sup>9</sup> histopathologic examination of mice sacrificed at intervals after injection revealed much depleted or aplastic bone marrow, whereas the spleens contained a remarkable increase in erythropoiesis. The fact that anemia failed to develop under these circumstances was considered to be directly related to the rapid development of an intense ectopic erythropoiesis in the spleens of the radiostrontium-treated animals. Accordingly, an experiment of greater scope, as described in this communication, was undertaken to determine the validity of this concept.

## MATERIALS AND METHODS

Young CF-1 female mice were divided into three groups of fifty or more animals each as shown in Table I. The mice in Group I were splenectomized, those in Group II received a single intraperitoneal injection of 2.0 microcuries of  $\text{Sr}^{90}$  per gram of body weight, and the mice in Group III were splenectomized and later injected with a dose of 2.0 microcuries per gram of body weight. A fourth group of animals not included in Table I, which was neither splenectomized nor given  $\text{Sr}^{90}$ , was used for base line hematologic controls and from this group animals were sacrificed at intervals to serve as normal reference for the histologic studies. Splenectomies were performed in Groups I and III while the animals were under ether anesthesia. Twenty-four days after splenectomy,  $\text{Sr}^{90}$  was administered intraperitoneally to the animals in Group III. The average weight of the mice at the time of injection was 20 grams. A second injection of 2.0 microcuries per gram was given to a representative number of animals in Groups II and III, 119 days after the original injection. Although a leucopenia of moderate degree persisted at this time in both groups (II and III), the animals in Group II which had been subjected to splenectomy and received  $\text{Sr}^{90}$  also had

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The photomicrographs were made by Jean M. Crunelle of the Photographic Department, Billings Hospital University of Chicago

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\*Raised by Carworth Farms homozygous for aa bb cc

TABLE I GENERAL SCHEMATA OF EXPERIMENT

GROUP	NUMBER OF ANIMALS	TREATMENT	PERIOD OF OBSERVATION AFTER ORIGINAL $\text{Sr}^{90}$ INJECTION
I	51	Splenectomy only	282 days
II	63	Injected intraperitoneally with $\text{Sr}^{90}$ in a dose of 2.0 microcuries per gram body weight	82 days
III	70	Splenectomy plus $\text{Sr}^{90}$ (2.0 microcuries per gram body weight)	282 days

Selected animals in Groups II and III were reinjected with a dose of 0 microcuries per gram body weight 119 days after original injection

recovered from the anemia. The purpose of the second injection of  $\text{Sr}^{90}$  was largely to determine whether or not compensatory ectopic erythropoiesis was sufficient in the splenectomized mice to prevent or minimize recurrence of anemia.

*Preparation of Radiostrontium ( $\text{Sr}^{90}$ ) Distribution and Excretion Studies*—As has been described elsewhere, the solution of radiostrontium was composed of  $\text{Sr}^{90}$  and  $\text{Sr}^{90}$ .  $\text{Sr}^{90}$  has a fifty-five day half-life and a maximum energy of 1.7 mev.  $\text{Sr}^{90}$  which represented 3 to 5 per cent of the initial stock solution on arrival from Oak Ridge has a half-life of circa thirty years and a maximum energy of 0.65 mev. The final preparation for administration was strontium chloride in isotonic solution. The greatest part of the radiostrontium excretion occurred during the first three days, and after ten to fifteen days virtually no more of the retained  $\text{Sr}^{90}$  was excreted. The average total excretion values were 34 per cent of the injected dose. Average total retention values were 45 per cent of the injected dose. By the third day after injection almost all of the retained  $\text{Sr}^{90}$  was deposited in the skeleton. Retention of  $\text{Sr}^{90}$  in the spleen was as follows:

First 24 hours	0.22 per cent/gram
24 to 72 hours	0.04 per cent/gram
3 days to 3 months	0.02 per cent/gram

In other soft tissues of the body the amount of radiostrontium per gram remaining after three days was well under 0.1 per cent of the injected dose.

*Hematologic Studies*—Studies of the peripheral blood were made on all four groups. These studies included determinations of the hemoglobin in grams per 100 ml (photoelectric), erythrocytes and leucocytes per cubic millimeter, leucocyte differentials (Wright's stain), and reticulocytes per cubic millimeter and in per cent (brilliant cresyl blue). Blood for study was drawn from the tail vein.

Control hematologic determinations were made on all animals after which splenectomy was performed on animals in Group I and III. Twenty-four days after splenectomy  $\text{Sr}^{90}$  was injected intraperitoneally into the animals in Groups II and III. Repeat hematologic determinations were made on all animals seven days after the original  $\text{Sr}^{90}$  injection and thereafter at twenty-nine day intervals. After the second injection of  $\text{Sr}^{90}$  in selected animals, hematologic studies were made at less frequent intervals.

*Histologic Studies*—Animals prepared as described were sacrificed at intervals through 280 days after  $\text{Sr}^{90}$  injection for histopathologic study. In addition, random animals in all groups on which hematologic studies were being conducted were sacrificed at various intervals. This latter procedure was followed in an attempt to correlate more closely the peripheral blood studies with the actual histopathologic state of the blood-forming tissue.

Tissues taken for study included skin, lung, small intestine (with Peyer's patch), adrenal, kidney, liver, spleen, thymus, lymph node, and bone marrow (femur and vertebrae). The tissues were fixed in Zenker's formol, embedded in 10 per cent nitrocellulose, sectioned at 8  $\mu$ , and stained with hematoxylin-eosin-azure II.

## RESULTS

*Hematologic Studies on the Peripheral Blood —*

(a) *Effect of Splenectomy Alone* The mean leucocyte value in the peripheral blood of splenectomized mice rose after splenectomy to stabilize between 14,000 and 22,000 per cubic millimeter during the period of observation (Fig 3). Splenectomy alone reduced the hemoglobin and erythrocyte values slightly during the first three weeks after splenectomy, but thereafter, as is shown in Figs 1 and 2, no appreciable fluctuation occurred.

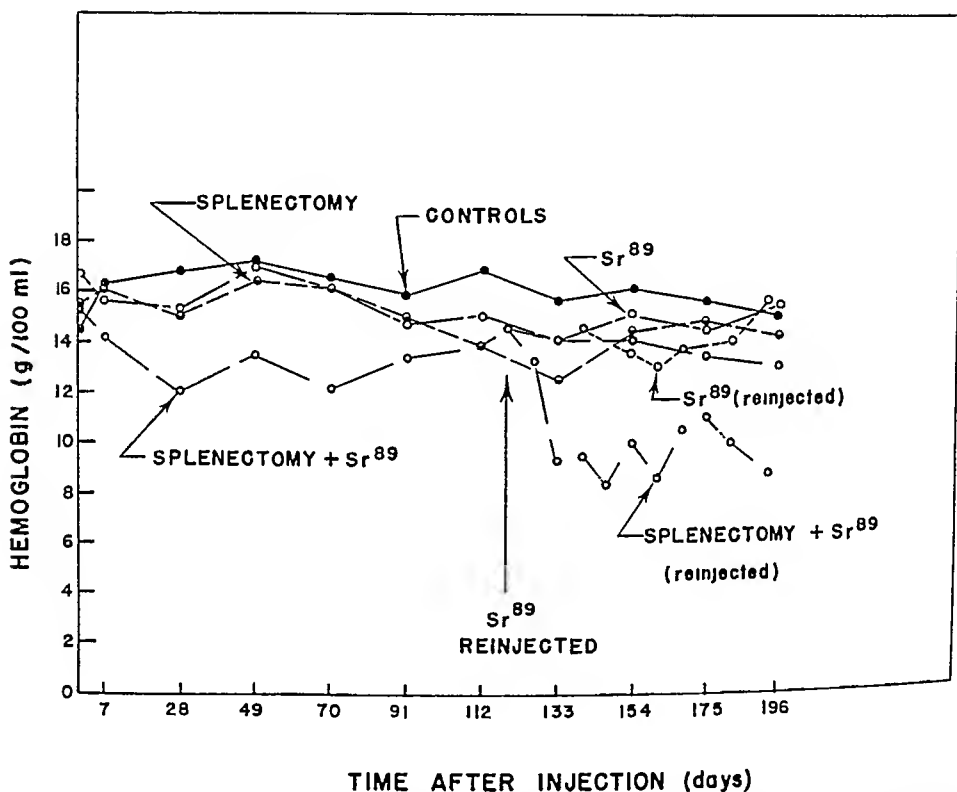


Fig 1—The effect of a single injection of 20 microcuries of radiostrontium per gram body weight on hemoglobin values of normal mice and mice splenectomized twenty-four days prior to Sr<sup>89</sup>. One hundred nineteen days after the original Sr<sup>89</sup> injection a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

(b) *Effect of Sr<sup>89</sup> Injection on Leucocytes of Peripheral Blood* The intraperitoneal injection of 20 microcuries of Sr<sup>89</sup> per gram produced a comparable degree of leucopenia in animals with an intact spleen and in animals that previously had a splenectomy (Fig 5). As is shown in Figs 4 and 5, the initial injection of Sr<sup>89</sup> reduced heterophils and lymphocyte values by 52 and 36 per cent, respectively, in twenty-eight days. The mean heterophil value of Sr<sup>89</sup>-injected animals which had intact spleens returned to the preinjection level in about forty-nine days and thereafter was actually higher than the

control value. The mean heterophil value of animals which had been splenectomized and were given  $\text{Sr}^{89}$  returned to the normal control or preinjection level in seventy days. The lymphocyte reduction after  $\text{Sr}^{89}$  injection was sustained in the animals with intact spleens and in splenectomized animals. In animals to which a second injection of  $\text{Sr}^{89}$  was given 119 days after the first injection, the reduction in heterophil and lymphocyte values was greater and more persistent in the splenectomized mice than in the intact mice (Figs 4 and 5).

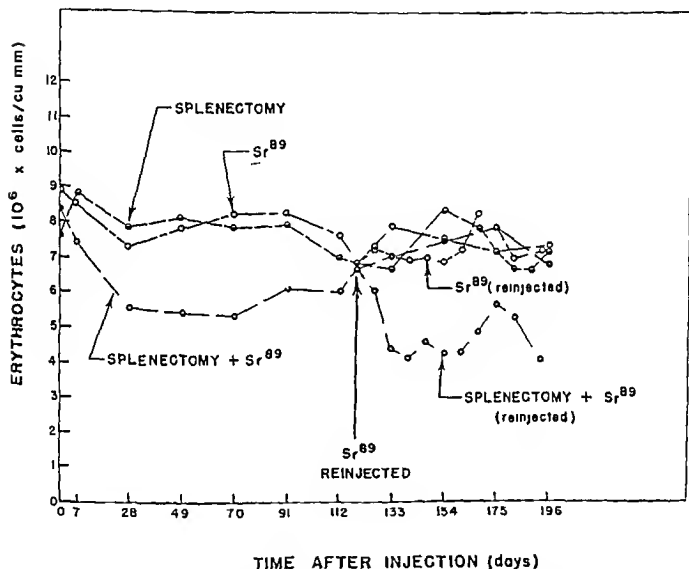


Fig. 7.—The effect of a single injection of 0 microcuries of radiostrontium per gram body weight on erythrocyte values of normal mice and mice splenectomized twenty four prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection a second injection of 0.2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

(c) *Effect of  $\text{Sr}^{89}$  Injection on the Hemoglobin and Erythrocyte Values of the Peripheral Blood* No anemia of significance was produced in mice with intact spleens to which  $\text{Sr}^{89}$  was given by intraperitoneal injection. On the other hand, splenectomized mice injected with 20 microcuries per gram developed a significant anemia in twenty eight days which persisted through seventy days, but with recovery in 119 days (Figs 1 and 2). Selected animals with an intact spleen and animals which had been splenectomized were given a second injection of  $\text{Sr}^{89}$  (20 microcuries per gram) 119 days after the

original injection As after the initial  $\text{Sr}^{89}$  injection, the mice with intact spleens developed no anemia, whereas the splenectomized group developed a precipitous reduction in both hemoglobin and erythrocyte values Although recovery from the anemia proceeded in the splenectomized group, the anemia recurred between the fifty-sixth and seventy-seventh day after the second injection at which time all the remaining animals died or were sacrificed for study (Figs 1 and 2)

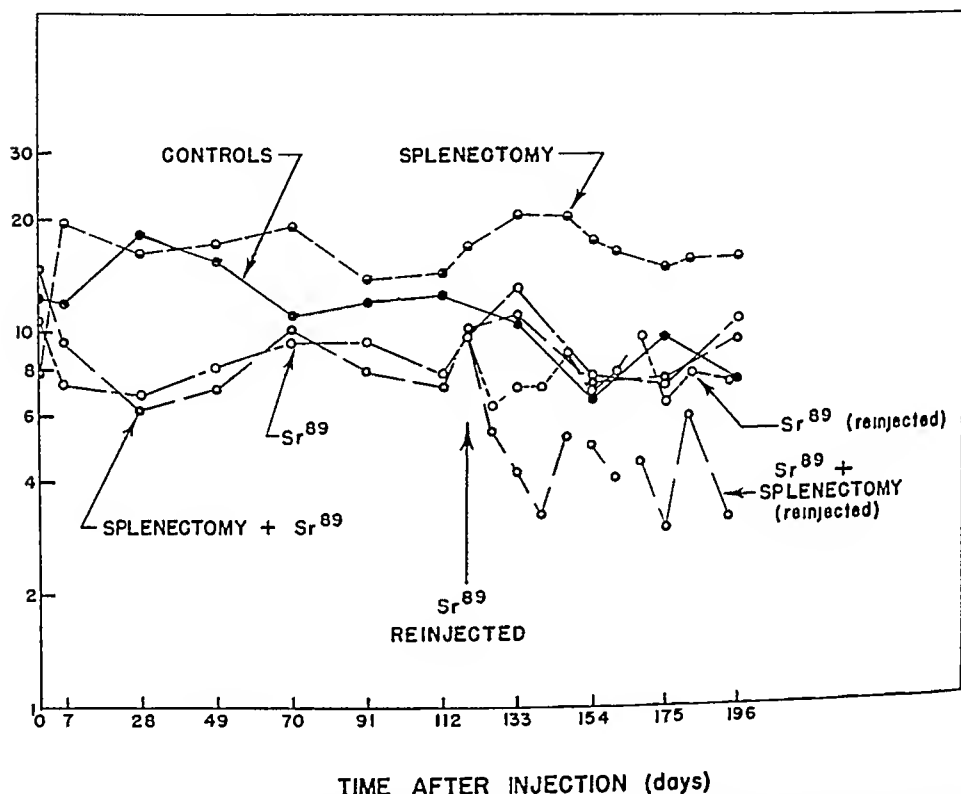


Fig 3—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on leucocyte values of normal mice and mice splenectomized twenty-four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope

(d) *Effect of  $\text{Sr}^{89}$  on Reticulocyte Values of the Peripheral Blood* Except for control studies no reticulocyte determinations were made until seven days after  $\text{Sr}^{89}$  injection, but at this point the reticulocyte values of animals which were splenectomized only and those which were given  $\text{Sr}^{89}$  only had actually risen, whereas the reticulocyte value of the group which was both splenectomized and strontium-treated had fallen significantly. Re-injection of  $\text{Sr}^{89}$  at 119 days produced no reduction in the reticulocyte value of mice with intact spleens. A reduction comparable with that seen after the original injection occurred, however, this was limited to splenectomized mice. As will be evident in the section on histology which follows, more frequent determinations



of the reticulocyte values of all the groups would have been of importance from the point of view of correlation

### Histopathologic Studies —

(a) *Observations on Lymphatic Tissue After Single Injection of  $\text{Sr}^{89}$*  No consistent difference in size of the spleen existed between  $\text{Sr}^{89}$  injected and control mice which were sacrificed during the 280 day period. However, an effect was observed in both the white and red pulp of the spleen in animals sacrificed three days after  $\text{Sr}^{89}$  treatment. This early effect consisted of a decreased cellularity of the white pulp and a marked hyperplasia of ectopic

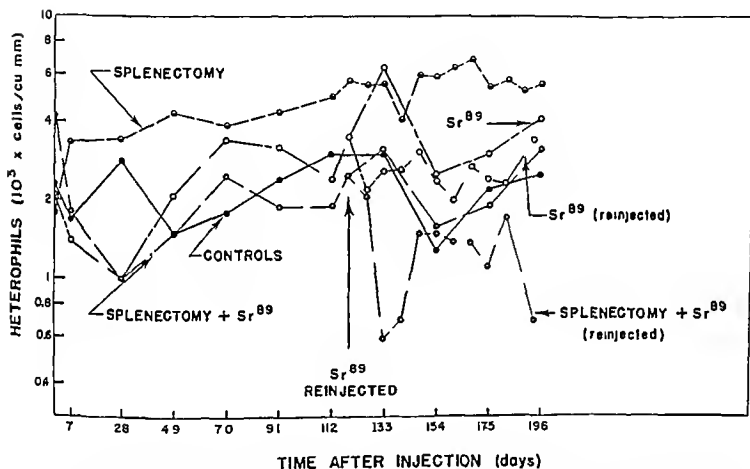


Fig 4—The effect of a single injection of 20 microcuries of radiostrontium per gram body weight on heterophil values of normal mice and mice splenectomized twenty four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

erythrocytopenia and megakaryocytopoiesis in the red pulp. Ectopic granulocytopoiesis was practically absent at this stage. Only a few scattered mature granulocytes could be found. There was little evidence of destructive effects, however, for there was no observable increase in pigment or debris-filled macrophages or pyknotic nuclei at this stage as compared with the spleens of control mice. The spleens of control mice usually contained small foci of ectopic erythrocytopenia, granulocytopoiesis, and megakaryocytopoiesis scattered about in the red pulp. The erythrocytopenia in the spleens of the  $\text{Sr}^{89}$  injected animals, however, was diffuse and intense at three days and consisted of predominantly large hemocytoblasts, basophilic erythroblasts, and a lesser number of more mature forms.

The number of young megakaryocytes in the spleens of  $\text{Sr}^{89}$ -injected mice three days after injection was greatly above that of the controls. This intense erythrocytopoiesis and megakaryocytopoiesis persisted in the  $\text{Sr}^{89}$ -injected animals at all the periods of examination through 280 days. Photomicrographs of the spleens of  $\text{Sr}^{89}$ -injected and control mice at intervals of three, twenty-nine, forty-five, and ninety-two days (Fig. 6) illustrate especially the

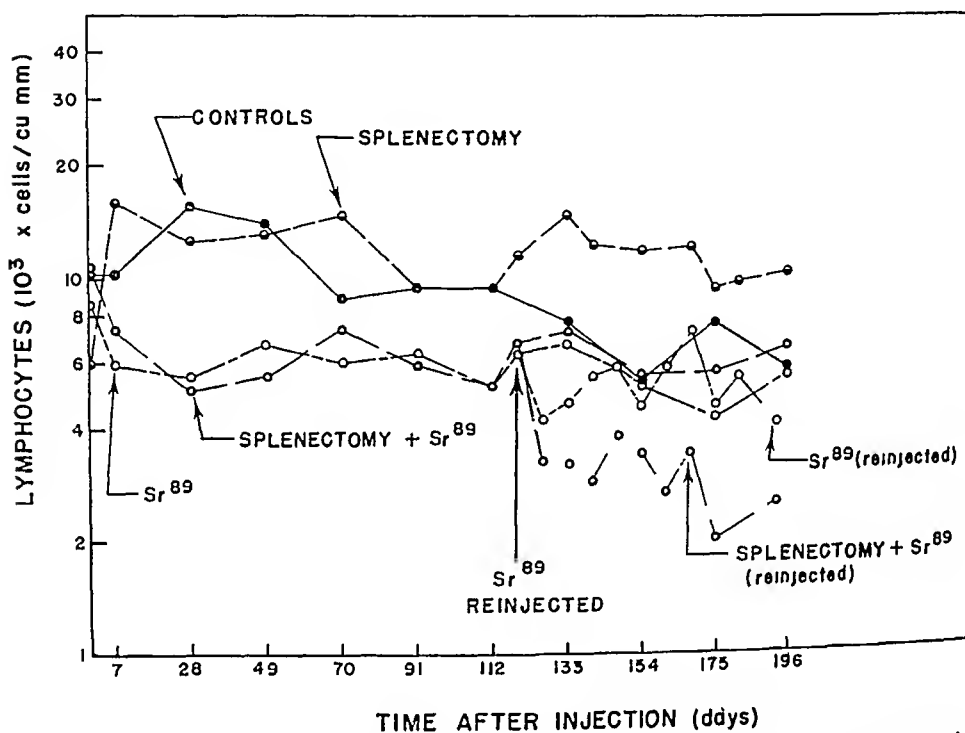


FIG. 5.—The effect of a single injection of 2.0 microcuries of radiostrontium per gram body weight on lymphocyte values of normal mice and mice splenectomized twenty-four days prior to  $\text{Sr}^{89}$ . One hundred nineteen days after the original  $\text{Sr}^{89}$  injection a second injection of 2 microcuries per gram body weight was given to selected animals from the two groups which originally were injected with this isotope.

degree of erythrocytopoiesis at these stages. Fig. 7 illustrates the closely packed erythroblasts in the red pulp at some of these stages at a slightly greater magnification.

No increase in ectopic granulocytopoiesis was observed in spleens of mice sacrificed at intervals of three and eight days after injection. Increased granulocytopoiesis became apparent in twelve days and increased to a maximum between forty-five and ninety-two days. In fact, in several of the mice examined during the forty-fifth and ninety-second days, granulocytopoiesis was qualitatively as intense as erythrocytopoiesis.

Changes in the white pulp in the spleens of  $\text{Sr}^{89}$ -treated mice were more or less universal at all stages studied from 3 to 280 days after injection. In normal mice the white pulp occupies circa 50 per cent of the spleen and, except for the distinct lymphatic nodules, blends gradually into the red pulp.

The splenic white pulp of the  $\text{Sr}^{90}$  injected mice almost without exception occupied less space than the red pulp and was distinctly separated from the intense erythroidopoietic and granulocytopoietic red pulp by a zone of relative acellularity consisting largely of reticular cells (Figs 6 and 7). The decrease in cellularity of the white pulp was observed especially in the early

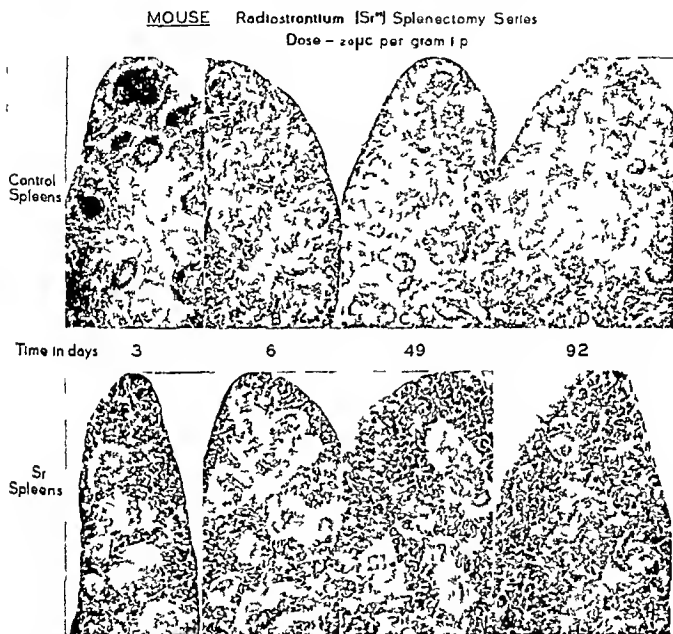


Fig 6—Photomicrographs of sections of the spleens of mice at intervals after  $\text{Sr}^{90}$  injection. The normal spleens show small areas of ectopic hematopoiesis in the red pulp and the normal variation in the white pulp, whereas the spleens of  $\text{Sr}^{90}$  injected animals show an intense ectopic hematopoiesis in the red pulp and a depletion of the cellularity of the white pulp (Hematoxylin-eosin azure II  $\times 15$ ).

stages after injection (i.e. three, six, twelve, fifteen, twenty one and twenty nine days), but the atrophy which persisted, is illustrated in the photomicrograph of the ninety second day specimen (Fig 7).

(b) *Lymph Nodes, Peyer's Patches of Intestine, and Thymus* Lymph nodes from the root of the mesentery and Peyer's patches from the small intestine from all animals sacrificed were studied in control and  $\text{Sr}^{90}$  injected groups. As opposed to the white pulp of the spleen of  $\text{Sr}^{90}$  injected animals in which atrophy, reduced cellularity and absence or diminution in the number of active lymphatic germinal centers were observed, the lymph nodes and

MOUSE      Radiostrophium [ $Sr^{90}$ ] Splenectomy Series  
Dose -  $\mu$ mc per gram l p

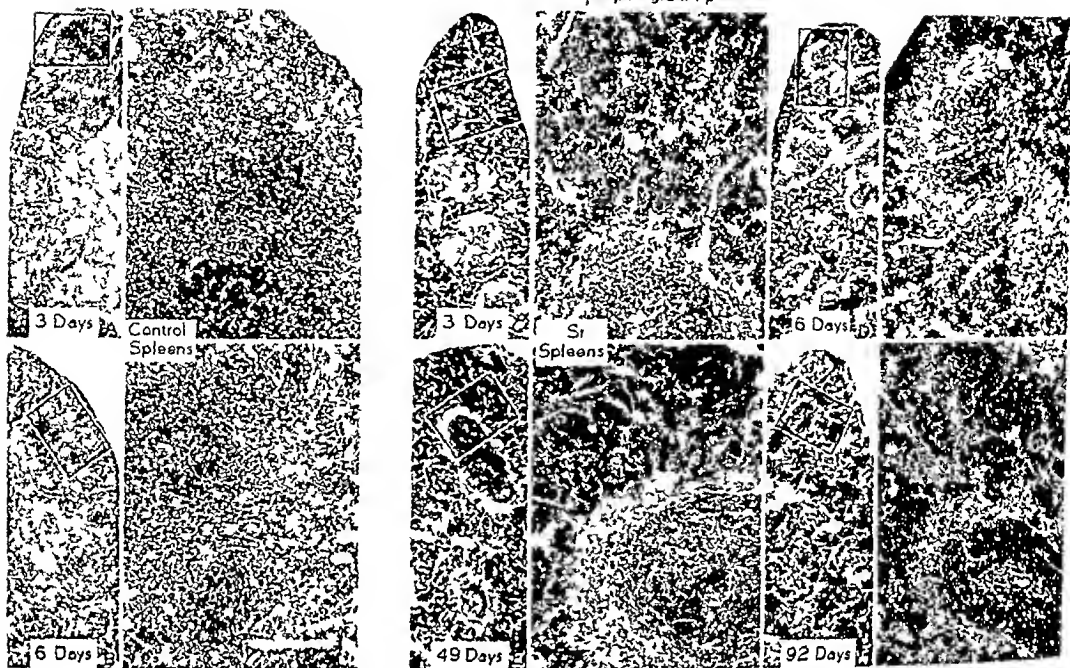


Fig 7—Photomicrographs of normal spleens and the spleens of  $Sr^{90}$ -injected animals with selected areas at a higher power to illustrate the intensity of the ectopic hematopoiesis in the red pulp and the depletion of cellularity in the white pulp of  $Sr^{90}$ -injected animals (Hematoxylin-eosin-azure II low power  $\times 15$  high power  $\times 70$ )

Peyer's patches appeared essentially normal and the germinal centers were active. Granulocytopoiesis, erythrocytopoiesis, and megakaryocytopoiesis were definitely increased in the medulla of the lymph nodes of some of the  $Sr^{90}$ -injected mice as compared with control mice. This increase in ectopic hematopoiesis in lymph nodes was observed as early as fifteen days after injection but was more prominent in later stages. No increase or decrease in plasma cells in lymphatic tissue was observed. No histologic changes which could be directly attributed to  $Sr^{90}$  were observed in the thymus.

(c) *Observations on Lymphatic Tissue After Second Injection of  $Sr^{90}$  at 119 Days* Only a relatively few stages were studied. These were at fifteen, twenty-four, and seventy-seven days after the second injection.

The histologic effects as far as lymphatic tissues were concerned were again limited to the spleen. The lymph nodes, Peyer's patches, and the thymus appeared essentially normal except for a moderate increase in granulocytopoiesis, megakaryocytopoiesis, and erythrocytopoiesis in the lymph nodes. At the time of the second injection, the erythrocyte and hemoglobin values were essentially normal in both groups of mice which had originally received  $Sr^{90}$  (Figs 1 and 2). The marked ectopic erythrocytopoiesis, megakaryocytopoiesis, and granulocytopoiesis, however, persisted in the spleens of the  $Sr^{90}$ -injected animals sacrificed at 119 days at a time comparable with

that at which selected animals received a second  $\text{Sr}^{90}$  injection. Although moderate atrophy of the white pulp was also apparent at this time active lymphatic germinal centers were present and in general the white pulp was more cellular than in earlier stages.

By the fifteenth day following the second injection of  $\text{Sr}^{90}$  ectopic erythropoiesis, megakaryopoiesis and granulopoiesis were more intense than at the 119 day stage. Ectopic granulopoiesis however was only moderate in degree. Again as after the first injection of  $\text{Sr}^{90}$  little evidence existed of active cellular disintegration in the form of pyknosis karyorrhexis, or phagocytosis. The white pulp which showed recovery to some extent after the original injection of  $\text{Sr}^{90}$  at 92 and 119 days in that it constituted a more normal percentage of the splenic tissue and was richer in medium and small lymphocytes, again was reduced in amount with a depletion of lymphocytes in the periphery of the white pulp fifteen days after the second injection. In the two other stages studied following the second injection namely twenty four and seventy seven days the recovery of the white pulp was only moderate, atrophy and decreased cellularity persisted.

As was true after the initial injection of  $\text{Sr}^{90}$  essentially "maximum" increased ectopic erythropoiesis and megakaryopoiesis were apparent fifteen days after the second granulopoiesis was progressively more intense at the twenty four and seventy seven day stages. Pigment filled macrophages scattered throughout the red pulp were increased in number at all stages studied after the second injection.

(d) *Observations on Bone Marrow After a Single  $\text{Sr}^{90}$  Injection* The observations made on bone and bone marrow are limited to the effects of  $\text{Sr}^{90}$  on hematopoiesis in the marrow spaces. A vertebra and a femur were taken for study from each animal sacrificed. Since the effects of  $\text{Sr}^{90}$  on vertebral or femoral marrow were essentially comparable in the mice with intact spleens and in those that were splenectomized prior to the injection, no attempt to differentiate between the two groups will be made in the description of marrow changes. In general the destructive effects of  $\text{Sr}^{90}$  on hematopoiesis were comparable in femoral and vertebral marrow in that if complete or partial atrophy were found in the entire femur the condition was also present in the vertebral bone marrow and vice versa. However moderate decrease in cellularity with or without fibrosis was not infrequently found in the metaphyseal region of the femur immediately adjacent to the epiphyseal cartilage without evidence of such localized changes in the vertebral marrow. The destructive effects of  $\text{Sr}^{90}$  and the subsequent depletion of the normal hematopoietic cells were most severe in the metaphyseal region of equal or slightly less severity in the epiphyseal marrow and least in the diaphysis of the femur. In some instances essentially complete depletion of normal hematopoiesis was found in the entire femoral and vertebral marrow spaces of mice sacrificed at fifteen through eighty days. In others although general cellularity of the femoral diaphysis and vertebral marrow space was reduced complete depletion was present only in the metaphyseal and epiphyseal regions of the bone.

In spite of the cellular depletion that occurred after  $\text{Sr}^{90}$  injection, no stage studied either in femoral or vertebral marrow showed clear histologic evidence of increased death of cells. The number of pyknotic nuclei and macrophages filled with particulate debris did not exceed that that was observed in control mice. A marked depletion of cells in the epiphyseal and

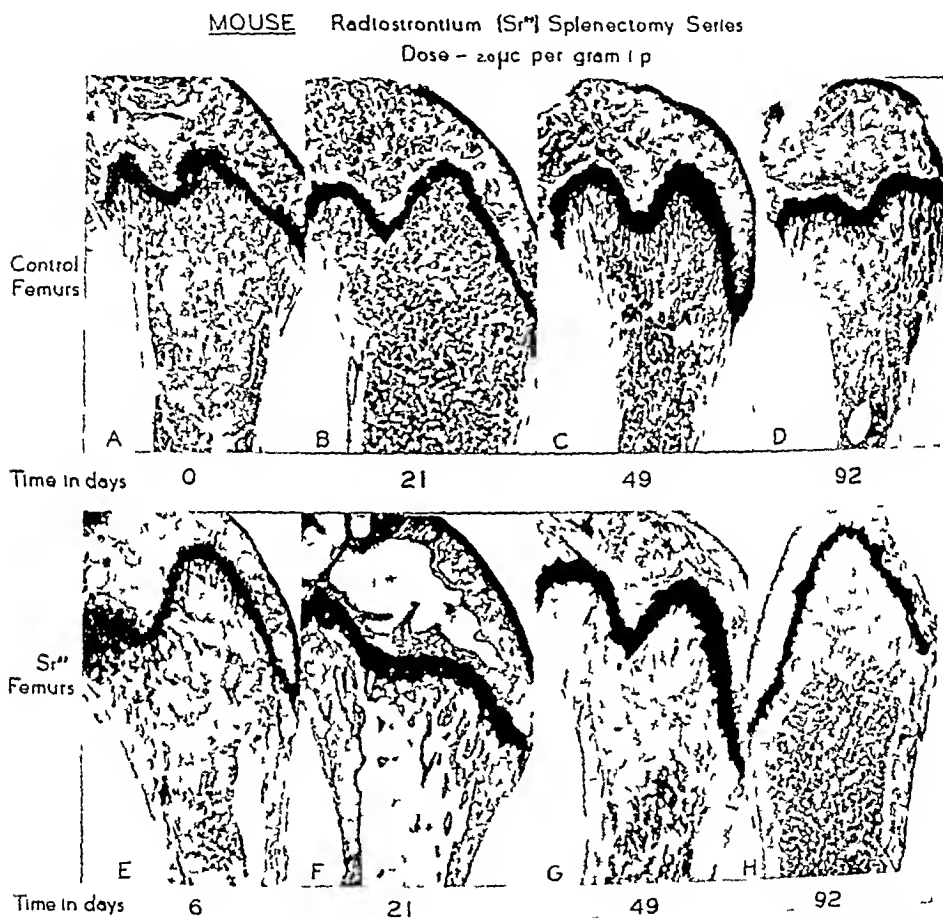


Fig 8—Photomicrographs of normal femoral marrow and of femurs of  $\text{Sr}^{90}$ -injected animals at various intervals after the  $\text{Sr}^{90}$  injection. These photomicrographs illustrate the depletion of normal hematopoietic cells in the epiphyseal and metaphyseal regions with partial sparing in the diaphysis of some animals. (Hematoxylin-cosin-azulc 11,  $\times 15$ )

metaphyseal marrow space of the femur was present three days after injection. The general cellularity of the diaphysis of the femur as well as in the vertebral marrow space was moderately decreased. In the epiphyseal and metaphyseal regions of the femur only a few scattered heterophils, plasma cells, and large lymphocytes remained at this stage, whereas dilated vascular spaces and increased fat cells filled the area. Distally from the depleted metaphyseal region, the cellularity of the marrow gradually increased. The diaphyseal marrow was diffusely cellular but distinctly less so than that of

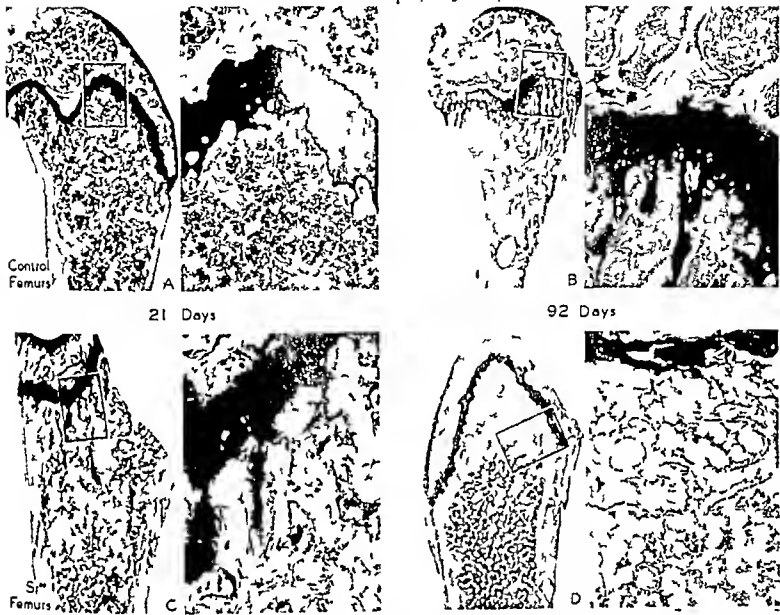
MOUSE Radiostrontium [ $Sr^{90}$ ] Splenectomy SeriesDose -  $2\mu\text{C}$  per gram i.p.

Fig. 9—Photomicrographs of normal femoral marrow and femurs of  $Sr^{90}$  injected animals with selected areas at a higher power to illustrate the loss of normal cellularity and replacement by fibrous tissue (C) twenty one days after  $Sr^{90}$  injection and the residual cellular depletion in the metaphysis ninety two days after  $Sr^{90}$  injection (Hematoxylin eosin azuro II low power  $\times 15$  high power  $\times 70$ )

normal control mice. The vascular spaces in this part of the marrow at three days were conspicuously dilated. megakaryocytes were fewer in number and erythrocytopoiesis was definitely decreased. The remaining cells were thus largely composed of the precursors of the granulocytic series.

The vertebral marrow was comparable with that of the femoral diaphysis at this stage. At six days the findings were essentially the same as those for the three day stage (Fig. 8 E). The degree of marrow depletion varied considerably from animal to animal at various time intervals after injection. In Fig. 8, F the femur of a mouse sacrificed twenty one days after injection is shown. In this specimen only fat and dilated vascular spaces filled with erythrocytes remained. In two other mice sacrificed at this same interval the epiphyseal and metaphyseal regions of the femurs were largely depleted of cells, the diaphyseal portions contained about as many hematopoietic cells as the controls. In still another mouse sacrificed at this interval the vertebral

and femoral marrow contained small areas of normal-appearing hematopoietic cells scattered about among the fat cells, dilated vascular spaces, and gelatinous areas, whereas the metaphyseal portion was more or less completely replaced by fibrous tissue (Fig 9, C). At later stages, i.e., 49, 71, 92, and 119 days after  $\text{Si}^{32}$  injection, the depletion of cells in the metaphyseal and epiphyseal portions of the femoral marrow remained conspicuous (Figs 8, G and 9, D). The shaft or diaphyseal portion of the femur and the vertebral marrow approached more normal hematopoietic activity. Likewise, in these stages the amount of fat and the dilation of the vascular spaces became less conspicuous.

(e) *Observations on Bone Marrow After Second  $\text{Si}^{32}$  Injection* Selected animals were given a second injection of  $\text{Si}^{32}$  119 days after the first injection. At this time (Figs 1 and 2) no anemia existed in either group which had originally received  $\text{Si}^{32}$ . Residual partial cellular depletion or fibrosis in the epiphyseal and metaphyseal region of the femur still persisted at this stage, but the diaphyseal portion of the femur and the vertebral marrow were only slightly less cellular than in normal mice. Sacrifices of mice that were given a second  $\text{Si}^{32}$  injection were made fifteen, twenty-four, and seventy-seven days after the injection. The effect on the marrow of these mice was essentially the same as that observed in comparable stages after the first injection except that the number of pigment-filled macrophages was slightly increased in the reinjected animals.

(f) *The Effect of  $\text{Si}^{32}$  on Other Tissues or Organs* The tissues or organs, not hitherto discussed, which were studied were skin, lung, adrenal, small intestine, kidney, and liver. No cytologic evidence of destructive effect which could be attributed to  $\text{Si}^{32}$  was noted in these organs or tissues. In an occasional animal, which had been given a single injection of  $\text{Si}^{32}$  or reinjection with a comparable dose of  $\text{Si}^{32}$ , ectopic erythropoiesis was found in the liver and adrenal. In each instance, however, this was minimal.

#### DISCUSSION

In spite of the extensive cellular depletion that develops in the bone marrow of mice injected with  $\text{Si}^{32}$  in a dose of 2 microcuries per gram of body weight, no anemia develops. Intense ectopic erythropoiesis occurs with such rapidity in the spleens of these mice that the steady state of the circulating erythrocytes remains essentially normal. Splenectomized mice, on the other hand, develop an anemia after the injection of this dose of  $\text{Si}^{32}$  because of the fact that compensatory ectopic erythropoiesis in the spleen is not possible and its development in other potential sites is not adequate. Increased ectopic megakaryocytopoiesis in the spleens of  $\text{Si}^{32}$ -injected animals occurred as rapidly as ectopic erythropoiesis, but since platelet determinations on the peripheral blood were not performed, no statement can be made with reference to whether or not this compensation was sufficient to prevent platelet reduction. In experiments suggested by the results related in this communication Jacobson and associates<sup>10</sup> showed that lead protection of the surgically mobilized spleens of mice during the administration of 600 r x-radia



tion to the balance of the body obviated the development of anemia and significantly reduced the severity and the duration of the leucopenia and thrombocytopenia which regularly follow the delivery of this dose to the whole animal. Histologic studies showed that this phenomenon was due to the rapid development of ectopic blood formation in the lead protected spleens.

An interesting problem that has been posed by this experiment relates to the fact that lymphocytopoiesis was markedly reduced in the spleens of  $Sr^{90}$  injected animals whereas the lymphatic tissue in the lymph nodes from the root of the mesentery, the thymus and in Peyer's patches of the small intestine was essentially unaltered at any stage studied. The amount of  $Sr^{90}$  found in the spleen of injected animals was minimal twenty four hours after injection and practically none was found three days after injection. It seems unlikely, therefore, that the reduction in lymphatic tissue in the spleen extending for more than ninety days after  $Sr^{90}$  injection could be attributed to the transient presence of  $Sr^{90}$  in the spleen. Neither can one explain this reduction in splenic lymphocytopoiesis adequately on the basis of radiation originating from  $Sr^{90}$  deposited in bone since one would expect other lymphatic tissue (lymph nodes, etc.) to be affected in a comparable manner. It would be difficult to explain the reduction in lymphatic tissue in the spleen on an indirect basis for the same reason. Although differences in the radiosensitivity of lymphatic tissue in various sites throughout the body of the mouse may exist, it seems unlikely that such an explanation is warranted here. The most plausible explanation for the decrease in splenic lymphatic tissue is that it may be related to a nutritional competition between the lymphatic tissue and ectopic hematopoiesis. The compensatory granulocytopoiesis in the spleen of  $Sr^{90}$  injected mice reached a maximum at a time approximately comparable with the time at which the heterophils in the peripheral blood returned to normal values (forty five to seventy days).

The delay in the appearance of ectopic granulocytopoiesis in the spleens of these animals suggests that this process was suppressed either primarily or secondarily, that the stimulus for an intensification of this process was less marked, or that even with a maximum stimulus the spleen had only a capacity for a gradual intensification of ectopic granulocytopoiesis.

The fact that anemia recurred in the splenectomized mice after the administration of a second injection of  $Sr^{90}$  at 119 days indicates fairly conclusively that ectopic erythrocytopoiesis in sites other than the spleen is wholly inadequate to cope with the destruction and suppression of erythrocytopoiesis which occurs in the bone marrow. The eventual recovery of splenectomized mice from the anemia induced by the first  $Sr^{90}$  injection must therefore have been largely a function of the recovery of erythrocytopoiesis in the marrow spaces.

The fact that histologic evidence of destruction in the bone marrow and spleen other than cellular depletion and the late appearance of pigment filled macrophages was not seen in  $Sr^{90}$  injected animals should perhaps be com-

mented upon. For example, as early as three days after  $\text{Sr}^{90}$  injection, partially to essentially complete depletion of hematopoietic cells in the bone marrow was seen. Only dilated vascular spaces, fat cells, a few debris-filled macrophages and occasional plasma cells, lymphocyte-like cells, etc., remained in the bone marrow of some of the animals sacrificed at this stage. It is likely that debris-laden macrophages, pyknotic nuclei, etc., would have been more in evidence had specimens been taken earlier than three days. Likewise, in the white pulp of the spleens of  $\text{Sr}^{90}$ -injected animals, active phagocytosis was minimal even though the general cellularity was markedly decreased in three days.

#### SUMMARY AND CONCLUSIONS

Radiostrontium ( $\text{Sr}^{90}$ ), a  $\beta$ -ray emitter with a half-life of fifty-five days, is largely deposited in the skeleton when administered to the experimental animal. Although deposition in bone is general, the greatest concentration of this isotope invariably occurs in areas of active bone growth.

A persistent leucopenia but no anemia of significance was produced in the peripheral blood of young mice given a single intraperitoneal injection of this isotope as the chloride in a dose of 2.0 microcuries per gram. The same dose produced a leucopenia and an anemia in splenectomized mice. Recovery from the anemia was essentially complete by 119 days after the  $\text{Sr}^{90}$  injection. Hematopoiesis was markedly reduced in the bone marrow of all radiostrontium-treated animals within three days after injection. The epiphyseal and metaphyseal regions of the long bones were largely depleted of cells, or, in some instances, the latter regions were completely replaced by fibrous tissue. Recovery of hematopoiesis in the bone marrow of the femur was essentially complete by 119 days except that some cellular depletion still existed in the metaphysis at this stage.

Ectopic erythrocytopoieses and megakaryocytopoieses were greatly increased in the spleen three days after  $\text{Sr}^{90}$  injection as was true of all other intervals studied through 119 days. Splenic lymphocytopoiesis, on the other hand, was reduced in three days and recovery was not complete by 119 days. Lymphocytopoiesis was essentially unaffected in other lymphatic tissues (lymph nodes, thymus, etc.). Ectopic granulocytopoiesis increased more slowly in the spleens of  $\text{Sr}^{90}$ -injected animals, reaching a maximum at circa sixty days. Ectopic granulocytopoiesis, megakaryocytopoiesis, and erythrocytopoiesis were not remarkable in other tissues of the animals which received  $\text{Sr}^{90}$ .

The rapid development and the persistence of ectopic erythrocytopoiesis in the spleen prevent anemia in mice given  $\text{Sr}^{90}$  intraperitoneally in a dose of 2.0 microcuries per gram.

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# JAPANESE B ENCEPHALITIS REPORT OF FIVE CASES

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## INTRODUCTION

THE epidemic of Japanese B encephalitis in Japan in the summer of 1948 was of significant interest because of the opportunity it afforded for direct observations on this exotic disease by physicians of the occupation forces. From a panoramic public health viewpoint, it was essentially of minor import as the actual percentage of the population afflicted was exceedingly small. In view of the rapidly expanding geographical confines of the disease, it may present a diagnostic and therapeutic problem to clinicians in the United States in the near future.

## EPIDEMIOLOGICAL AND ETIOLOGICAL ASPECTS

The purpose of this article is to point out the clinical observations on a small series of five serologically proved cases observed at the Navy Dispensary, Yokosuka, Japan. Nevertheless it does not seem amiss to discuss some of the features of the epidemiological and etiological aspects.

The geographical distribution of the disease is rather widespread, cases having been reported from Okinawa and the near-by islands, Korea, China, Guam (epidemic of 1947), Siberia, the Japanese islands, and possibly many other countries of the Far East.<sup>1 2 3</sup>

**Vectors**—In our present state of knowledge, it is believed that the encephalitic virus is conveyed by a mosquito. Inada,<sup>4</sup> a Japanese investigator, and two Russian workers, Petrisheva and Shubladá,<sup>5</sup> have reported the isolation of the virus from *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens*. Animal reservoirs of infection have been discovered in horses, pigs, goats, cows, and possibly sparrows, but not in chickens.<sup>1, 2</sup> It is interesting to note, as demonstrated in studies in Japan, that outbreaks among domestic animals may occur coincident with no or few human clinical cases.<sup>6</sup> Presumably epidemic outbreaks may occur only when the factors of mosquitoes, animal reservoirs of infection, and human susceptibles exist concomitantly. The disease has presented a fluctuating course in Japan, reaching epidemic or minor epidemic proportions every few years, a feature probably related to the accumulation of susceptible persons. In July, 1924, there were 6,125 cases with a mortality rate of 62 per cent. In 1935, there was an outbreak of 5,370 cases.<sup>7</sup> One interesting aspect of the epidemic under discussion is that the conditions of temperature and rainfall were exceedingly favorable for the propagation of mosquitoes prior to the outbreak of the disease.

**Incidence in Man**—Apparently, from evidence accumulated from many sources, the clinically recognizable cases represent only a relatively small

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proportion of the total the majority smoldering, under the blanket of subclinical or very mild clinical infection. In 1936, one year after a large epidemic, 83 per cent of 116 sera tested in Tokyo revealed virus neutralizing antibodies.<sup>1</sup> In 1937, 0.8 per cent of 525 human sera obtained from Hokkaido, Japan, demonstrated these antibodies.<sup>2</sup> Hokkaido is not an epidemic nor an endemic area of the disease. In 1941, 83 per cent of 104 sera from Middle China possessed neutralizing ability against the Japanese B virus,<sup>3</sup> and in 1945, 90 per cent of the natives of Okinawa similarly had neutralizing antibodies.<sup>1</sup> In 1946, a similar phenomenon was found in the sera of 85 per cent of thirteen Chinese residents of Shanghai and 89 per cent of nineteen Chinese residents of Tientsin.<sup>3</sup>

Sabin<sup>1</sup> reported a mortality rate for the 1945 outbreak on Okinawa of 28.6 per cent and on the nearby islands of Hainan and Hamahika 30.6 per cent. Among American military forces on Okinawa in 1945 there were two deaths occurring in a series of twelve severe cases. Figures compiled by the Public Health and Welfare Section, Supreme Commander for Allied Powers, revealed that in the first forty-one weeks of 1948 there were approximately 8,000 cases of Japanese B encephalitis throughout the empire and 2,374 deaths were reported. The approximate mortality rate from the disease was 25.7 per cent for the Tokyo area and 35.7 per cent for all Japan. In the summer of 1948 there were three fatalities in the twenty-nine proved cases of Japanese B encephalitis in occupation personnel in Japan. Because of the paucity of serologically proved cases occurring among occupation personnel in Japan, a study of the features of the disease as manifested in the five cases forming the subject of this paper seemed all the more desirable.

Although superficially the number of deaths in 1948 attributable to the encephalitic virus seems fairly large, it shrinks considerably when viewed relative to the 78 millions inhabiting the Japanese islands. These fatalities fade almost into oblivion when one contemplates the staggering total of approximately 147,000 deaths in Japan in 1947 directly attributable to tuberculosis. According to several attending Japanese physicians, approximately 2 per cent of the patients exhibit sequelae. These include clonic muscular movements, decerebrate rigidity, and twitchings of muscle groups.

The figures for the incidence in the Tokyo area in 1948 reveal a high rate for the younger age group, i.e. through 1 to 20 years of age. The highest occurred in the 6 to 10 years group, the rate being 117.3 per 100,000. The lowest incidence occurred in the 31 to 70 years group, the rate being 9.1 per 100,000. In the early years, male patients have the highest incidence of the disease, but this tendency decreases after the age of 20 until there is a female preponderance in the 51 to 60 years group. In all ages, the mortality among female patients is higher than that among male patients. The overall case fatality rate in Tokyo during the 1945 epidemic was 19.9 per cent for males and 33.3 per cent for females. These figures were derived from 2,067 cases.

Two cases, occurring in nonvaccinated Naval personnel, are of interest in that they assist in elucidating the time requisite for the incubation period of the

disease. Two firemen from a Naval vessel stayed ashore overnight in Yokohama. The ship remained in Yokohama for two days, sailing Aug. 1, 1948. Many mosquitoes were observed aboard ship. On August 11 one man reported to the ship's sick bay with a complaint of headache of four hours' duration. He seemed drowsy and had a fever. The patient was transferred to a British hospital at Ceylon thirty hours later and died twenty-four hours after transfer. Unfortunately, no autopsy was performed. On August 21 the other fireman developed a headache and fever and became disoriented. A spinal fluid examination performed at a hospital at Bahrein Gulf showed a slight pleocytosis and an increase in protein. The patient's clinical course was similar to that of cases of Japanese B encephalitis occurring in Japan. Following complete recovery, the man returned to Japan. His complement-fixing titer one month after the original infection was 1:16, and the neutralization index was 63,000.

*Status of Vaccination*—The status of vaccine has not been established at this time. No conclusions relative to the efficacy of vaccination could be drawn from the epidemic on Okinawa. Sabin<sup>9</sup> has shown a rise in neutralization and complement-fixing antibodies in young people inoculated with mouse brain vaccine, two 1 cc doses being given six days apart and the last dose of 1 cc one month later. Very elderly persons who had no previous serologic neutralizing antibodies exhibited no response. Following the administration of a potent vaccine, many persons develop a rise in neutralizing antibodies.<sup>7</sup> In American adults, no rise in complement-fixing antibodies occurs as a result of vaccination alone, although a person previously infected with the live virus may develop complement-fixing antibodies after vaccination.<sup>7</sup> The vaccine used at present is a chick embryo vaccine, and its efficacy is under intense investigation.

Twelve patients of the twenty-nine cases occurring among occupational personnel in Japan had received complete immunization. No deaths occurred among this group. Three patients had received complete immunization except for one dose. No deaths occurred among these. Six patients had failed to obtain complete vaccination by two or more doses. There was one death among them. Two deaths occurred among eight completely nonimmunized patients.

*Symptoms*—The clinical symptoms of the disease as previously reported by Sabin<sup>1, 2</sup> include marked lethargy, confusion, high fever, nuchal rigidity, leucocytosis, and spinal fluid pleocytosis. The onset is abrupt, with sudden headache and fever. Sabin, in his Okinawa series<sup>1</sup> (ten serologically proved), observed convulsions in only two cases, both of them fatal. He stressed the duration of the fever, seven to eleven days, and the constant bradycardia present. Cells in the spinal fluid were predominantly mononuclear, whereas a polymorphonuclear leucocytosis was found in the peripheral blood smear. One patient of his series exhibited interesting sequelae, i.e., hyperirritability, mask-like facies, somnolence, and a propensity for urinating on the floor. In the Korean outbreak, two instances of bladder paralysis were recorded. One of these patients died. In the other, bladder function became normal upon convalescence.

*Serologic Studies*—Serologic studies upon the Okinawa cases revealed interesting data. Serum neutralizing antibodies were questionably or certainly positive on the fourth or fifth day of the disease. Low titers of complement fixing antibodies were found as early as two to eight days after the onset. However, these complement fixing antibodies may not appear until the fifth week. No discrepancies were noted between the results of the neutralization and complement fixing tests. For several technical reasons Sabin feels that the complement fixing test is more practical. It is interesting that many cases, initially suspected of being mild or abortive cases in his Okinawa series gave no serologic evidence of Japanese B encephalitis.

*Pathology*—Gross pathology reveals a diffuse congestion of the brain, sometimes with softening of the cervical cord. There is diffuse involvement of the leptomeninges and the gray matter. Perivascular lymphocytes are predominant in the arachnoid meshes. The meningeal lesions are widespread over the surface of the brain, being minimal over the midbrain, pons, medulla, cerebellum, and spinal cord. Many nodules composed of mononuclear cells, clustered about degenerating ganglion cells or blood vessels occur in the gray matter. The cortex, thalamus, substantia nigra, nuclei of the fourth ventricle, cerebellar cortex, and the anterior horns of the spinal cord are involved. Adrenal "tubular degeneration" may occur.

#### CASE REPORTS

**CASE 1**—Patient T. H., a 23 year old white man entered the Navy Dispensary on Aug 8, 1948, complaining of a headache beginning the previous day. This headache began suddenly progressively became worse and was accompanied by malaise and chills. He had completed the course of immunization against Japanese B encephalitis approximately two months before the present illness.

*Physical Examination and Laboratory Data* T 102.4 F, P, 84, R, 22, BP, 116/78 mm Hg. The patient appeared flushed, dehydrated and acutely ill. There was definite nuchal rigidity. The reflexes were physiologic and Kernig's sign was negative. The spinal fluid showed 30 polymorphonuclear cells and the Pandy reaction was negative. Blood leukocyte count on the second hospital day was 10,900 with 76 per cent polymorphonuclear cells. Admission urinalysis was negative. Initial chest films, as well as all subsequent chest films were negative.

*Course* Intravenous fluids, oral sulfadiazine and intramuscular penicillin were instituted. On the second hospital day the fever rose to 104 F. There were 162 white cells in the spinal fluid, of which 127 were polymorphonuclear. During the next two days the temperature climbed to 104.6 F, and there was no essential change in the neurological picture. The patient was relatively alert. On the fourth hospital day the spinal fluid contained 133 polymorphonuclear and 98 mononuclear cells, and the Pandy reaction at this time was 2 plus. The patient became completely disoriented on the fifth hospital day. He did not respond to questioning, had to be restrained in bed and was unmanageable. Paraldehyde was necessary for sedation. There were definite neurological changes. The arms developed a lead pipe type of rigidity, and there were marked gross tremors of the upper extremities and tongue with twitchings of the mouth. On the sixth hospital day, the temperature suddenly dropped and remained normal on the seventh day. The patient became more rational. A lumbar puncture at this time revealed approximately 400 cells in the spinal fluid, about three fourths of them being polymorphonuclear, and a two plus Pandy reaction. Improvement after the seventh hospital day was rapid, and the temperature remained essentially

normal During the subsequent thirteen days, the mental cloudiness, muscle twitching, and muscle tremors disappeared as well as all traces of nuchal rigidity However, the patient's voice, which had become quite husky, remained so for two months This has cleared completely at the time of the present writing, i.e., approximately five months from the onset of the illness When the patient became ambulatory again, he complained bitterly of dizziness when walking, but this complaint rapidly disappeared

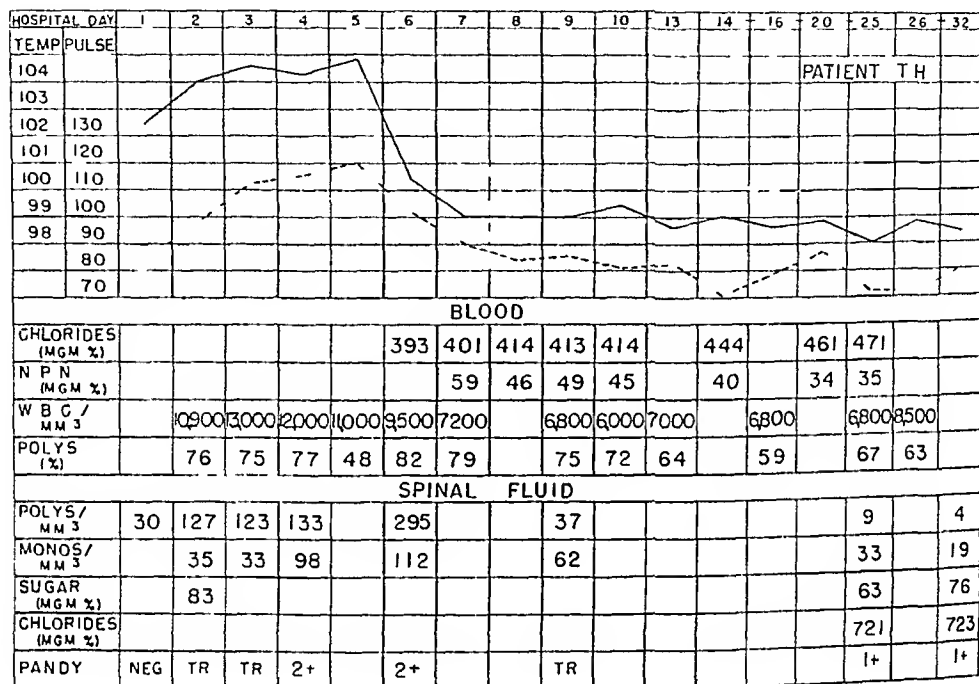


Fig 1

CASE 2—Patient M B, a 37 year old white man, entered the Navy Dispensary on Aug 28, 1948 The patient stated that the evening before he had experienced sudden severe head ache and chills He remembered starting to the Dispensary the next morning, but affirmed later that he did not have any recollection of his arrival nor of the immediately subsequent events There had been no immunization against Japanese B encephalitis

*Physical Examination and Laboratory Data* T, 103.2° F, P, 96, R, 22, BP, 142/78 mm Hg Within a few moments after admission to the medical ward, the patient experienced a severe clonic convulsion with attendant cyanosis The convulsion lasted approximately five minutes, and upon its cessation the patient was unruly and completely irrational, necessitating restraint by several men The patient was disoriented and could make no intelligent reply to questioning The administration of intravenous Sodium Amytal was necessary There was flushing of the face and extreme dehydration A foul discharge, from which pneumococci were subsequently cultured, drained from a right otitis externa The mucous membranes of the throat were injected, and the tongue had been traumatized during the convulsion A Grade II apical systolic murmur was present There was marked nuchal rigidity Kernig's sign was positive All reflexes were hypoaactive, the abdominal and ankle reflexes were absent, and the Babinski was positive bilaterally The spinal fluid contained 425 cells, of which 321 were polymorphonuclear cells The Pandey reaction was two plus The initial blood leucocyte count was approximately 14,000 with 82 per cent neutrophils The urine contained a trace of protein The chest film was negative



*Course* Therapy, in the form of oral sulfadiazine intramuscular penicillin intravenous fluids, and intranasal oxygen was instituted. On the second hospital day the patient developed a lead pipe rigidity of the arms and gross tremors of the lips and upper extremities. Similar but less marked rigidity of the lower extremities was also evident. This clinical picture persisted for six days. On the seventh day the temperature dropped to 99.8 F. The gross tremors of the lips, tongue and arms were still present. The ankle jerks

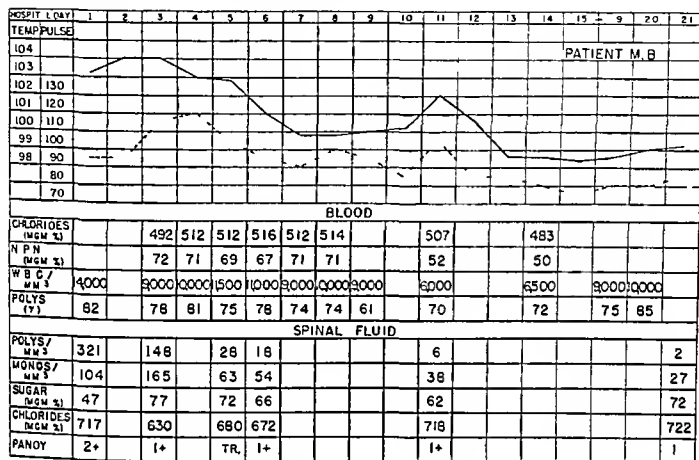


Fig. 2

were absent the Babinski was negative and all other reflexes were hypoactive. Transient weakness of the external ocular rectus muscles appeared. It was necessary to discontinue oxygen for approximately thirty minutes because of a mechanical defect in the apparatus and at the end of that time it was noted that the slightest stimulus could precipitate violent movements bordering on a convulsion. Following resumption of oxygen inhalation for a few minutes the patient became much more responsive and much less hyperirritable. After the eighth hospital day he became much clearer mentally and rapidly regained his normal sensorium. Likewise, the muscle tremors cleared. Neurological abnormalities disappeared and no physical sequelae of the encephalitis remained. His convalescence was complicated by a spondylitis.

**CASE 3**—Patient W. P., a 24-year-old white man entered the Navy Dispensary on Aug. 19, 1948, complaining of headache, fever, malaise and nuchal rigidity of two days' duration. He had been immunized against Japanese B encephalitis in 1946 and had received a booster dose of 1 cc in 1947 and in June 1948.

**Physical Examination and Laboratory Data** T 104 F 110 R 24 BP 1—/—4 mm Hg. The patient was acutely ill and markedly dehydrated. The skin was red and flushed. There was slight stiffness of the neck and transient weakness of the external rectus muscles of both eyes. All reflexes were normal and the Babinski signs were normal. Initial spinal fluid studies revealed 143 white cells, 107 of them being polymorphonuclear and a one plus Pandy reaction. The blood leukocyte count was 10,000 with 53 per cent polymorphonuclear cells. The urinalysis was negative. Initial and subsequent chest films were normal.

CASE 5—Patient M W, a 29 year old white woman, entered the Navy Dispensary on Aug 19, 1948, complaining of fever, malaise, and headache. These symptoms had begun suddenly three days before admission. The evening before admission she had become disoriented and irrational. There had been no immunization against Japanese B encephalitis.

*Physical Examination and Laboratory Data* T, 105.5° F, P, 120, R, 24, BP, 110/80 mm Hg. The patient was flushed and dehydrated. She was irrational and could not respond to questioning. A gross tremor of the upper extremities and twitching of the mouth

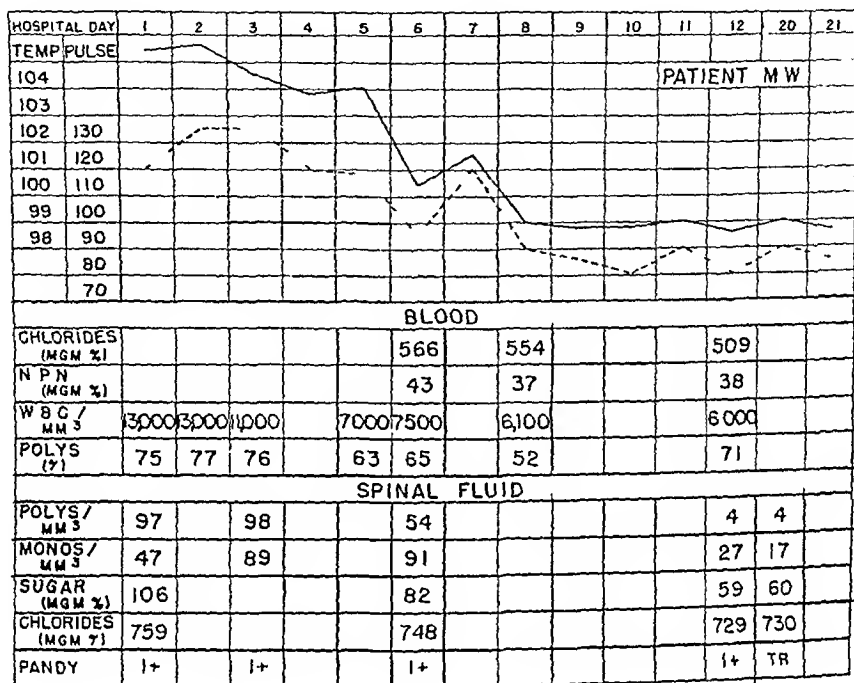


Fig 5

were present. Definite nuchal rigidity could be elicited. There was mild injection of the mucous membranes of the nose and throat. All reflexes were physiologic except for a questionably positive Babinski on the right. There were 97 polymorphonuclear cells and 47 mononuclear cells in the spinal fluid. The Pandy reaction was one plus. The blood leukocyte count was 13,000, 75 per cent of these cells being polymorphonuclear. The urinalysis was negative. Initial and subsequent chest films were negative.

*Course* Intravenous fluids, oral sulfadiazine, and intramuscular penicillin were administered. During the following four days the patient remained delirious and continued to have the gross tremors described. Lead pipe rigidity of the upper extremities also appeared. On the sixth hospital day the temperature decreased to 100.5° F and on the seventh hospital day the patient became much clearer mentally. Thereafter improvement was rapid and all neurological abnormalities quickly disappeared. No sequelae remained.

#### DISCUSSION

The diagnosis in each of the five patients was proved by a rise in complement-fixing antibodies in the serum. The results of the complement fixation tests and the neutralization indices are shown in Table I.

Six other patients, having the same type of symptoms, spinal fluid pleocytosis, and clinical picture, are omitted from this discussion because positive serologic proof was not obtained. The question arises as to whether

TABLE I COMPLEMENT FIXATIONS AND NEUTRALIZATION INDICES

PATIENT	HOSPITAL DAY	COMPLEMENT FIXATION	NEUTRALIZATION INDEX
Case 1 T H	20	1 16	320
	26	1 64	1 000
	35	1 64	10 000
	†	1 64	2 000
	‡	1 32	10 000
Case 2 M B	6	Neg	030
	17	1 8	20 000
	21	1 32	2 000
Case 3 W P	2	1 4	—
	9	1 4	10 000
	15	1 32	10 000
	26	1 32	200 000
	35	1 64	320 000
Case 4 J S	†	1 32	10 000
	2	1 64	0 300
	9	1 256	13 000
	14	1 32	13 000
Case 5 M W	2	Neg	1 000
	9	Neg	3 200
	15	Neg	3 200
	21	1 8	10 000

1 A titer over 1 4 is considered significant

† Follow up twelve days after discharge

‡ Follow up five months after onset

these were indeed cases of Japanese B encephalitis and whether it is possible that in some instances the threshold of immunologic response is not reached

The therapeutic regimen adopted was necessarily varied to compensate for the individual needs of the patient. One of the characteristic features of the disease was the marked dehydration present initially. The nonprotein nitrogen determinations were elevated in two patients even after several days of rehydration and in a third before rehydration was accomplished. Probably the nonprotein nitrogen would have been elevated in the other two patients had this value been determined at the beginning of their hospitalizations.

Blood chloride values were generally within normal limits, and blood nonprotein nitrogen determinations were consistent with the degree of dehydration evidenced by the patient. In no patient could it be demonstrated that renal disease was a factor in the elevation of the nonprotein nitrogen. A few fasting blood sugar determinations were normal. Several blood calcium determinations in two patients experiencing convulsions were normal.

Large quantities of saline and glucose were administered intravenously until adequate oral intake could be assured. As these individuals were quite young large quantities could be infused with relative safety. In all cases adequate urinary flow was achieved. In no instance did edema occur except in one patient who developed pulmonary edema following repeated convulsions. This edema quickly responded to morphine.

Intravenous barbiturate sedation was essential in two men to control multiple convulsions. Paraldehyde was requisite for one man who had become aggressive and irrational.

Sulfadiazine and penicillin were instituted in all patients because at that time, the exact nature of the disease had not been ascertained. It is

doubtful whether this therapy altered the course of the disease itself in any way. However, since among the Japanese bronchopneumonia was a common complication after the lysis of the encephalitic fever, chemotherapy may have had some value in the prevention of secondary infection.

One of the most valuable therapeutic adjuncts was oxygen. Many observers have expressed the opinion that the pathologic changes demonstrable at autopsy are to a large degree contingent upon anoxia. The salutary effect of oxygen was seen definitively in one patient. Patient M. B., Case 2, was receiving intranasal oxygen. After his initial convulsion had been controlled with Sodium Amytal and intranasal oxygen had been started, no further convulsions occurred even in the absence of further sedation. At one period in the illness, oxygen was discontinued for thirty minutes because of a mechanical defect in the apparatus. At the end of this period of time, the patient became much more restless and more delirious and upon slight stimulation developed convulsive movements which threatened to culminate in a generalized grand mal type of attack. Intranasal oxygen was then reinstituted, and after fifteen minutes the patient could respond to some questions intelligently, the hyperirritability of the central nervous system had subsided, and even strong stimuli failed to elicit convulsive movements. Oxygen was again discontinued temporarily, this time purposefully, and in half an hour the patient had reverted to his former state of lethargy and hyperirritability. Thereafter oxygen was administered continuously, and the clinical response was swift and gratifying. It is interesting to note that oxygen was almost specific when there were absolutely no signs of circulatory distress. The chest and heart were normal clinically and roentgenologically, and no clinical cyanosis nor respiratory distress was apparent.

A second patient, Patient W. P., Case 3, already receiving oxygen, experienced recurrent convulsions and developed pulmonary edema. Intravenous Sodium Amytal controlled the convulsions, and a hypodermic injection of morphine controlled the pulmonary edema. Thereafter, with intranasal oxygen, in the absence of further sedation, the patient remained calm and free of convulsions. The role of oxygen in controlling the convulsions in this case is less clear-cut than in that of Patient M. B., Case 2, but it was difficult to escape the clinical impression that oxygen was at least partially responsible in the suppression of convulsions. In retrospect, oxygen probably should have been administered to all patients.

Headache was a very distressing symptom. Frequently the simple procedure of withdrawing 10 cc of spinal fluid for study ameliorated the headache considerably. All of the patients had slight elevations of the spinal fluid pressure. Aspirin and codeine were valueless. Some clinicians of the occupation forces employed intramuscular caffeine and sodium benzoate with good results. We had no experience with this method of treatment.

Incontinence of urine and feces was a problem only so long as the patient was comatose.

The remainder of the therapy was purely symptomatic and similar to that employed in any acute infectious disease. Respiratory and intestinal isolation

techniques were instituted, but it is dubious whether the disease is contagious via the respiratory route. Those persons entrusted with the care of encephalitic patients in Japanese hospitals practiced no precautions and seem none the worse for their multiple exposures. Throughout Japan there are extremely few instances of the disease occurring in more than one member of a family. The necessity of adequate mosquito screening seems obvious.

Many of the features of the disease can be discerned readily by a brief study of the charts. Necessarily, from such a small series no sweeping conclusions can be drawn. These charts represent only the clinical picture in five patients seen at this Dispensary. Good correlation has been obtained, however, with much larger series occurring among Japanese nationals throughout the empire.

Initially, all patients were febrile, the height of the elevation ranging from 102° F. to 105° F. plus. Definite lysis of the fever occurred on the seventh day of the illness in two patients, on the eighth and ninth days in two others, and on the tenth day in one. Temperature lysis about the seventh day of the illness is characteristic of Japanese B encephalitis. It is interesting to attempt to correlate the degree of the febrile response with the prevailing disturbance of the sensorium. Four patients had markedly disturbed sensoria. The fifth, Case 4 Patient J S, with an equally high temperature and with the most marked spinal fluid pleocytosis, was mentally alert and cheerful throughout his illness.

Relative bradycardia was present initially in only two patients. This quickly disappeared.

The blood leucocyte count varied from 10 000 to 15 000 on admission and in the majority of these counts there was a decided 'shift to the left'. The count returned to normal in three to seven days.

Pleocytosis in the spinal fluid ranged initially from 30 to 552 cells. In four patients there was a preponderance of polymorphonuclear cells. In the fifth, no differential was done on admission. However, the next day the majority of cells in the spinal fluid were polymorphonuclear. The ratio of neutrophilic leucocytes to mononuclear cells was soon reversed and subsequent spinal fluid examinations revealed a decided majority of mononuclear cells. The Pandy reactions were positive semiquantitatively from a trace to two plus. There were no strongly positive Pandy reactions in any stage of the disease. Spinal fluid sugars and chlorides were normal in all instances. Repeated smears of the centrifuged spinal fluid were negative for organisms. No bacterial growth was obtained on repeated cultures. Because of the difficulty in following these patients after their discharge from the Dispensary and in order to avoid repeated lumbar punctures, it was impossible to ascertain exactly the length of time requisite for all abnormalities to disappear from the spinal fluid. In the five patients definite abnormalities were found on the tenth, twentieth, twenty first, thirty second and thirty second days respectively.

During the febrile episode there was transient, mild proteinuria, but this finding disappeared upon lysis of the fever.

The neurological picture during the course of the illness has been described in the case histories. Nuchal rigidity was present in all, although the rigidity was quite mild. In only one patient, Case 3, Patient W P, did a cranial nerve palsy develop (seventh nerve), although a transient weakness of the external (lateral) ocular rectus muscles was present in Case 3, Patient W P, and in Case 2, Patient M B. All except one, Case 4, Patient J S, experienced gross tremors of the upper extremities, lips, and tongue. It is noteworthy that the Japanese physicians consider a tongue tremor to be one of the first signs of recovery. This observation was not well correlated with the clinical course in this series. The ocular fundi were normal in all patients. Reflex changes were varied and transient. At various stages of the illness there were hyperactive, hypoactive, and absent knee jerks, ankle jerks, and abdominal jerks. Kernig's sign was positive in some but not all patients. The Babinski was transiently positive in some. There was rapid disappearance of neurological reflex abnormalities after lysis of the fever. All patients, except Case 4, Patient J S, exhibited a lead-pipe type of rigidity of the upper extremities. The sensoria of all patients, except Case 4, Patient J S, were markedly deviated from normal. These patients were restless and aggressive, with alternate periods of lethargy. This is in marked contrast to the extreme constant lethargy usually prevalent among native encephalitis victims.

One characteristic of the disease which deserves special emphasis is the rapidity of the patient's recovery. He may appear moribund, and the outlook may seem virtually hopeless. Even in such instances recovery may be swift.

There were no fatalities in this small series. The Japanese mortality rate of approximately 35.7 per cent is undoubtedly excessively high because of the poor methods of treatment in their hospitals. Even during the acute stage, while the patient is comatose, only 500 cc of fluid are administered each day by intramuscular drip, according to the practice in one of their large hospitals. This amount is not varied, regardless of the degree of dehydration, age, or size of the patient. "Acidosis" is "prevented" by the intravenous administration each day of 20 cc of a 50 per cent glucose solution. Oxygen, apparently, is infrequently, if ever, employed. A further disadvantage is the marked overcrowding of their hospitals with encephalitic patients, rendering very difficult the task of individualized attention by the staff of doctors.

Two patients in this series developed minor physical sequelae. In one, Case 1, Patient T H, there was a definite change in the voice. The patient developed a very hoarse voice which improved after two months. No paralysis of the vocal cords has been demonstrated. The possibility of weakness of the superior laryngeal nerve was entertained. A second man, Case 3, Patient W P, developed weakness of the adductor of the left thumb and flexors of the left forefinger. Initially, sensory perception was lost over the dorsal and palmar aspects of the second and third fingers of the left hand. The sensory abnormalities disappeared, leaving the residual muscle weakness outlined. Physiotherapy has greatly improved this condition.

The possible permanent mental changes resulting from infection with the encephalitic virus would comprise a most interesting and fruitful field for study. Limitations of time and lack of trained psychiatric consultants pre-

vented a detailed investigation of this important aspect of the disease. Lack of knowledge concerning the behavioristic pattern of the patient prior to his illness represents an intrinsic impediment in a study of this type. Without proper controls only gross abnormalities can be attributed to the disease. None of these patients exhibited any marked impairment of the process of cerebration. Their intellectual ability was well within the normal range when tested by simple measures such as arithmetic problems, ability to exercise judgment in simple situations and general knowledge.

Emotionally a similar pattern of dependence was evident in three of them. Even when the storm of the illness had passed and the patients were alert and recuperating remarkably, there was a strong reluctance on their part to attend in any way to their own needs. Only with difficulty, with the exception of one patient (Case 1, Patient T H) were they persuaded to abandon their recumbent positions. They were very loath to accept again the responsibility of looking after themselves. One patient (Case 2 Patient M B) with a probable spondylitis was extremely happy in his prolonged convalescence and almost refused to attempt anything for himself except the actual eating of his meals. However, the remainder of the patients did bow to the inevitable and, with one exception, Case 5 Patient M W, were eager to return to their occupations. Case 5 Patient M W was anxious to return home provided she could convalesce for an indefinite period. Later it was ascertained that she promptly installed herself in bed at home and there remained for six weeks. The patient manifested extreme dependence, emotional lability and was excessively demanding of those who attended her. According to one of her friends she had definitely "changed" since her illness. Lapses in recent memory became obvious. Upon one occasion while entertaining guests her husband left the room for a few moments. Almost coincident with his departure his wife confided in a friend her anxiety concerning him as he had failed to return home that evening and had not called. Obviously with no adequate pre-confinement studies personality tendencies cannot indisputably be ascribed to the Japanese B virus.

One man (Case 1, Patient T H) who least displayed this type of personality dependence, developed a curious propensity for inserting himself into odd and dangerous situations. He had a definite proclivity for wandering through the women's ward at night. He was content to confine his wanderings to the corridors and never did he enter any of the rooms. He had no explanation for his behavior. In one instance he stopped the elevator between floors, climbed on top and was reclining comfortably when discovered. Again, there was no adequate explanation. However, these bizarre quips of behavior disappeared rapidly, and the patient was quite embarrassed when they were recalled to his attention. Interestingly enough, these peculiar phenomena occurred when the man seemed alert and well oriented. It seems possible that the encephalitic damage occasioned these transitory abnormalities.

All of the convalescent patients were unusually cheerful. Even when residues such as focal nerve palsy or tremors were evident the patients ignored them magnanimously and refused to allow themselves to sink into the depths of depression. Only one patient (Case 5 Patient M W) was emotionally labile,

but even she usually maintained a cheerful equilibrium. This emotional lability persisted in her for approximately six weeks after discharge.

The routine course of immunization comprised the administration of 1 c c of the chick embryo vaccine at weekly intervals for two doses followed by an additional 1 c c one month later. Case 4, Patient J S, had only two injections, Case 2, Patient M B, had none, Case 1, Patient T H, had completed his course of immunization, Case 3, Patient W P, had been immunized in 1946 and had received a booster dose of 1 c c in 1947 and 1948, Case 5, Patient M W, had received no immunization.

#### SUMMARY

1 A series of five serologically proved cases of Japanese B encephalitis is presented. Six other patients with the same signs and symptoms were hospitalized during the epidemic but are not included in the series as no positive serologic proof could be obtained.

2 Only two of the five patients had completed their immunizations against Japanese B encephalitis.

3 The clinical features are outlined.

4 In addition to rehydration and sedation when necessary, oxygen seems to be a valuable therapeutic adjunct.

5 Only two minor physical sequelae occurred in five cases. There were no deaths.

6 The possibility of personality changes, resulting from the encephalitic virus, is discussed.

7 Improved treatment will probably reduce considerably the high mortality rate for Japanese B encephalitis prevalent among the Japanese.

The author wishes to express his appreciation to the Virus and Rickettsial Section, 406th Medical General Laboratory, Tokyo, Honshu, Japan, for the determination of complement fixations and neutralization indices.

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## PANCREATITIS IN INFECTIOUS MONONUCLEOSIS

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PANCREATITIS in association with mumps is often mentioned, though the literature on this point is not conclusive. Pancreatitis has been demonstrated definitely, however, in three patients with mumps, one at necropsy<sup>1</sup> and two at the time of surgical exploration.<sup>2</sup>

There are few autopsy reports of cases of infectious mononucleosis. In eight case reports<sup>3-5</sup> the pancreas was not mentioned. In three case reports<sup>6-10</sup> the pancreas was described as normal. There seems to be general agreement that infectious mononucleosis is a disease which may affect many organs.

Lucke and Mallory<sup>11</sup> in their excellent study of the pathology of hepatitis did not mention the pancreas. Capps<sup>1</sup> however mentions lesions occurring in the intestinal tract and in the pancreas. Comfort<sup>12</sup> noted in one case a persistent steatorrhea after infectious hepatitis and speculated on the association of pancreatitis with hepatitis.

In order to explore further the possibility that pancreatitis may be associated with other viral diseases, determinations of serum amylase and lipase were made at approximately weekly intervals on twenty consecutive patients with infectious mononucleosis and nine patients with viral hepatitis.

### MATERIALS AND METHODS

Serum amylase was determined by the Somogyi method.<sup>14</sup> Incubation times were determined to the nearest minute. The range of error in duplicate and triplicate determinations of the same specimen of normal serum was 0 to 36 per cent with a mean error of 6 per cent. Serum lipase was determined by the Cherry-Crandall<sup>15</sup> method as modified by Maclay.<sup>16</sup> Titrations were done with 0.1N NaOH to the nearest 0.1 cubic centimeter. The range of error on duplicate and triplicate determinations on the same specimen of normal serum was 0 to 50 per cent with a mean error of 14 per cent. The mean control amylase value based on thirty-six normal subjects was 146.4 units with a standard deviation of 55.7. Using three times the standard deviation as a criterion the upper limit of normal for this group would be 313.5 Somogyi unit. The mean control lipase value based on thirty-seven normal subjects was 0.27 cc with a standard deviation of 0.16. Using three times the standard deviation as a criterion the upper limit of normal for this group would be 0.75 cc of 0.1N NaOH.

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## RESULTS

Two of the twenty patients with infectious mononucleosis presented abnormal serum enzyme values. In one patient values for both serum amylase and serum lipase were elevated, the highest serum amylase being 450 Somogyi units and the highest serum lipase being 17 c.c. of 0.1N NaOH. The second patient had abnormal values of serum lipase, the highest being 10 cubic centimeter. In this instance the values for serum amylase, although within the normal range, increased from 108 to 228 units. Liver function tests were done in nineteen of these patients and suggested an associated hepatitis in eighteen.

In nine patients with viral hepatitis, similar enzyme determinations at various stages of the disease gave normal results in all instances. Three were considered to be of the infectious type and six to be of the homologous serum variety.

Because of their particular interest, the two patients with infectious mononucleosis who, during the course of their illness, exhibited elevated serum enzyme values suggestive of an associated pancreatitis are presented in some detail (Table I).

CASE 1—A 24 year old, white, male school teacher, who had been in good health previously, was admitted to the hospital Jan. 3, 1948, complaining of headache and malaise of fourteen days' duration and of sore throat and fever up to 103° F for one week. Penicillin and sulfadiazine had been administered by his family doctor without benefit. Physical examination revealed an acutely ill patient, the temperature being 103.4° F, and the presence of cervical, inguinal, and axillary adenopathy, marked tonsillitis, hepatomegaly, and splenomegaly. The hemoglobin concentration was 12.6 grams per 100 c.c. of blood and the leucocytes numbered 20,800 per cubic millimeter of blood. The percentages of the various types of leucocytes were as follows: neutrophils 30, lymphocytes 66 (many of them atypical), monocytes 2, basophils 1, and eosinophils 1. The Kahn test was 2 plus. Albuminuria was graded 2 plus and microscopic examination of the urine sediment revealed 3 to 6 leucocytes, occasional erythrocytes, hyaline casts, and granular casts per high power field. Roentgenogram of the chest revealed moderate accentuation of the bronchovascular markings bilaterally and a faint suggestion of pneumonitis in the left lower lung field. Bromsulphalein retention was 10.5 per cent at 45 minutes using a dose of 5 mg. per kilogram per body weight. The result of the cephalin flocculation test was 3 plus in 24 hours and 3 plus in 48 hours. The one minute serum bilirubin was 0.2 mg. per cent and the total was 0.5 mg. per cent. Predominantly hemolytic staphylococci grew from a throat culture.

A clinical impression of infectious mononucleosis was corroborated by the relative and absolute lymphocytosis, the appearance of atypical lymphocytes in the peripheral blood smear, and a strongly positive heterophile antigen antibody agglutination titer. The laboratory studies are presented in detail in Table I.

On the thirty first day of illness the serum amylase was 450 units and the serum lipase was 17 cubic centimeters. In approximately twenty days these values had returned to normal. A glucose tolerance curve at the height of the elevation of serum amylase and lipase was normal.

Treatment consisted of bed rest, administration of 50,000 units of penicillin intramuscularly every three hours for the five days after admission, a high carbohydrate, low fat, and high protein diet with vitamin supplements and added brewers' yeast. The Kahn and Kolmer Wassermann tests were negative after the patient had been two days in the hospital. The abnormal urinary findings disappeared after one week. A roentgenogram of the chest, taken ten days after admission, was normal. One week after penicillin therapy was discontinued a maculopapular rash appeared over the trunk. The liver was enlarged and tender for approximately one month after admission. There was never any abdominal

TABLE I DETAILED CLINICAL AND LABORATORY DATA OF THE TWO PATIENTS WITH INFECTIOUS MONONUCLEOSIS WHO HAD ELEVATED VALUES OF SERUM AMYLASE AND LIPASE

DATE (1948)	DAY OF DISEASE	TEMPERATURE	RASH	TONSILLITIS	WBC X 1 000	PER CENT POLYS	PER CENT LYMPHO CYTES	HETERO PHILE TITER	CEPHALIN FLOCCULATION	SERUM BILI RUBIN	BROM SULF ALEIN (%)	RAHA	SERUM AMYLASE	SERUM LIPASE
Case 1														
Jan 3	15	103	-	3+	129	31	64	1 1492				2+		
Jan 6	18	102	-	2+	208	30	64		3+ 3+				450	14
Jan 8	20	101	-	2+									400	13
Jan 11	23	100	-	1+	165	10	86	1 1707			100	N <sub>64</sub>		
Jan 15	27	99	+	-								N <sub>64</sub>		
Jan 21	31	98.6	+	-	94	23	75		3+ 3+	2-5				
Jan 23	33	98.6	+	-										
Jan 26	36	98.6	-	-	84	26	73	1 1492					200	14
Jan 28	38	98.6	-	-									46	10
Jan 29	39	98.6	-	-										0
Feb 4	44	98.6	-	-										
Feb 10	51	98.6	-	-										
Feb 13		98.6	-	-	97	32	93						130	
Case 2														
Apr 4	5	102	-	-	80	61	34							
Apr 6	7	102	-	-	73	47	44	1 448						
Apr 8	9	102	-	1+	117	29	65		3+ 3+	100			105	
Apr 12	13	103	-	2+	168	30	60	1 896						
Apr 14	15	102	-	-					3+ 4+					
Apr 19	20	100	-	-					3+ 4+					
Apr 21	22	98.6	-	-									158	10
Apr 22	23	98.6	-	-										0.9
Apr 23	24	98.6	-	-									28	0.6
Apr 26	27	98.6	-	-									200	
Apr 28	29	98.6	-	-									190	0.5
June 10		98.6	-	-					0 1+				120	

pain or diarrhea but occasionally the patient complained of backache. Appetite and strength gradually returned and the lymphadenopathy decreased. During the course of the illness the patient lost 23 pounds in weight.

CASE 2—A 21 year old, white student nurse who had previously been in good health was admitted to the hospital April 4, 1948, complaining of a slight cold and nasal congestion of five days' duration. Two days before admission she had noticed fever, fatigue, chills, sensations, and loss of appetite. Physical examination revealed an acutely ill patient with a temperature of 102° F. There was a slight mucoid nasal discharge. The throat was normal in appearance. The heart and lungs were within normal limits. A slightly enlarged, tender lymph node was found in the right anterior cervical region. The liver and spleen were not enlarged and there was no rash.

The hemoglobin concentration was 12.6 grams per 100 cc of blood and the leucocytes numbered 8,000 per cubic millimeter. The percentages of the various leucocytes were as follows: neutrophils 61, lymphocytes 34, monocytes 3, and eosinophils 2. The Kahn test for syphilis was negative. The urinalysis did not reveal abnormalities. Roentgenogram of the chest was normal. *Streptococcus viridans* and *Neisseria catarrhalis* grew from a throat culture. The heterophile antigen antibody titer was 1:448. The cephalin flocculation test gave 3 plus results in 24 hours and 48 hours. The one minute and total serum bilirubin concentrations were 0.2 and 0.5 mg per cent respectively and the bromsulfalein retention with a dose of 5 mg per kilogram of body weight was 11 per cent. Alkaline phosphatase was 10 King Armstrong units. The prothrombin time was 44 per cent of normal. Total proteins were 6.2 grams per cent with 3.3 grams per cent of albumin and 2.9 grams per cent of globulin. The blood cholesterol was 134 mg per 100 cc of serum with 42 per cent cholesterol esters. Bleeding time, clotting time, and platelet counts were normal. Two routine blood cultures and one blood culture with increased carbon dioxide tension were sterile. The blood was type O and Rh negative.

On the ninth day of illness the serum amylase was 108 Somogyi units and on the twentieth day, 158 units. On the twenty second day the serum lipase was 10 cc of 0.1N NaOH and on the twenty third day was 0.9 cubic centimeter. On the twenty fourth day the serum amylase and lipase were 228 units and 0.6 cc respectively. Subsequent values for serum lipase rapidly returned to normal and the serum amylase decreased to 120 units. At this time the fasting blood sugar was 96 mg per cent and the urinalysis for sugar was negative.

Subsequent development of an absolute lymphocytosis, the appearance of atypical lymphocytes in the stained peripheral blood smear, and a strongly positive heterophile antigen antibody titer established the diagnosis of infectious mononucleosis. See Table I for complete details of the laboratory studies.

On the tenth day of disease numerous petechiae were noted on the hard palate which persisted approximately four days. About the same time the normal pharynx became injected in appearance, the tonsils were covered by a grayish white membrane, and at this time the predominant organism on throat culture medium was nonhemolytic streptococcus. The sore throat accompanied by headache and pain on moving the eyes had nearly disappeared when, on the fifteenth day of illness, the patient became markedly anorexic and nauseated and vomited repeatedly. Treatment at this time entailed administration intravenously of solutions containing glucose, Parenamine, B complex vitamins, ascorbic acid, and Synkavite. The liver became tender to percussion but still was not enlarged. The patient became afebrile on the twenty second day of illness and by the twenty ninth day she was sufficiently improved to be discharged to her home for gradual resumption of activities. She had lost at least 8 pounds in weight.

#### SUMMARY AND CONCLUSIONS

1. Twenty consecutive cases of infectious mononucleosis were studied at weekly intervals and in two instances a definite elevation of the values of serum amylase and lipase was demonstrated. This finding is suggestive of an as

sociated pancreatic disturbance which to our knowledge has not been described previously in patients with infectious mononucleosis. This was possibly a pancreatitis, but lymph node obstruction of the pancreatic ducts must be considered.

2 In nine cases of viral hepatitis the serum amylase and lipase determinations were made at varying intervals and no elevated values were found. No conclusions can be drawn from this small series except that there was no evidence for pancreatitis at the time the determinations were made. This series should be extended and the possibility of pancreatitis occurring in other viral diseases should be explored.

3 It is quite possible that many asymptomatic acute subacute and chronic inflammatory changes of the pancreas exist in association with viral diseases which at the present time are not appreciated. It is conceivable that such infection may play a role in the etiology of chronic relapsing pancreatitis, the pathogenesis of which remains obscure.

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## A CASE OF CONGENITAL IDIOPATHIC METHEMOGLOBINEMIA

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THE differential diagnosis of cyanosis in the adult does not often present a difficult problem except in the instance of chronic, persistent cyanosis which does not vary. This is more interesting if the patient is middle aged or older, and if the cyanosis has been present during the greater part of his life. Proper consideration must be given to cyanosis produced by altered hemoglobin compounds within the circulating blood. The nature of the compound and its concentration in the blood are important in the diagnosis, treatment, and prognosis of these disorders. The three pigments (always intracorporeal) which most frequently produce cyanosis are reduced hemoglobin (as in cardiopulmonary disease), methemoglobin, and sulfhemoglobin. When these are present in the blood in concentrations of 5, 15, and 0.5 Gm. or less per 100 ml. of blood, respectively, cyanosis may be clinically evident.<sup>1</sup> Minute amounts of methemoglobin (0.03 to 0.13 Gm. per 100 ml.) have been reported in the blood of normal persons.<sup>2</sup> This may be a normal stage in the degradation of the pigment. The compounds have several definite chemical and physical properties by which they may be identified.<sup>3</sup>

Clinically, methemoglobinemia may be primary or secondary. The secondary type is more common and is most often due to drug toxicity. Anemia and/or granulocytopenia are usually accompanying findings in chronic cases, and the systemic effects are striking. Of much less frequent occurrence is the primary type. There is no anemia, indeed, a polycythemia is often present apparently as a compensatory measure. Systemic symptoms are exceptionally mild, and a familial incidence has been noted in many of the cases reported. Since the condition is rare, the following case is believed to be of interest.

### CASE REPORT AND EXPERIMENTAL STUDY

A 61-year-old white man was admitted to the Marine Hospital with complaints of failing vision in both eyes. On physical examination, bilateral cataracts were discovered. It was also noted that the patient had a marked cyanosis of the mucous membranes, lips, and fingers, and a slate gray color of the skin all over the body. There was no clubbing of the digits, and the conjunctival and retinal blood vessels were engorged. The patient stated that his blue gray skin coloring had been present "since birth," and that people had frequently remarked about it when he was a child. No other familial instance could be elicited. There was no history of prolonged drug ingestion, and system review failed to disclose any abnormality of gastrointestinal function. The patient had no complaints other than his visual disturbance, and cardiopulmonary disease was ruled out by physical examination, x-ray and fluoroscopic studies, and normal electrocardiographic tracings.

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The patient was seen in consultation by Dr. A. J. Beams, who suggested a diagnosis of idiopathic methemoglobinemia. A sample of blood was examined and found to contain total hemoglobin 21.9 Gm. per 100 ml. oxyhemoglobin 14.3 Gm. and the difference 7.6 Gm., was nonfunctional hemoglobin which showed a spectroscopic absorption band similar to that of a known methemoglobin solution. Laboratory findings at this time revealed nothing of significance except an icterus index of 17.6 units, red blood cells, 6,350,000 per cubic millimeter, and a hematocrit of 65 per cent packed red cell. The red cell indices revealed a mean corpuscular volume of 106 cmm. and mean corpuscular hemoglobin concentration of 29.2 per cent. The white blood cells were normal.

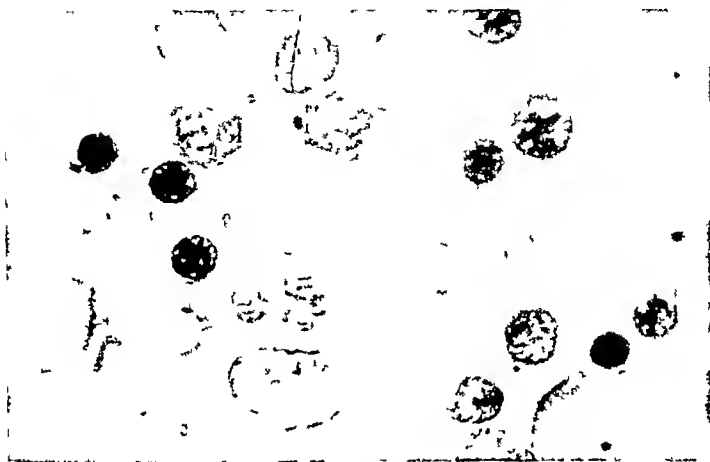


Fig. 1—Sternal bone marrow ( $\times 950$ ) showing normoblastic hyperplasia.

The patient was operated on for a cataract of the left eye and then discharged from the hospital. Four months later he was asked to return to the hospital and was admitted to the medical service for further study of his methemoglobinemia. On this second admission he had no complaints and was not dyspneic. The physical examination was essentially the same as on the first admission except for a partial left iridectomy and absence of that lens. The blood pressure was 150/94, temperature 98.2 F, pulse 80, and respirations 24. The red blood cell count was 5,930,000 per cubic millimeter with a hemoglobin of 18.9 grams. The hematocrit was 61 per cent. When venous blood was drawn it had a dark chocolate brown color. Exposure of the blood to air over a period of twenty-four hours produced no noticeable change in color, and long periods of oxygen inhalation did not affect the patient's cyanosis. The reticulocytes numbered 0.7 per cent and the icterus index was 13.0 units. The total hemoglobin measured 19.9 Gm., oxyhemoglobin, 12.5 Gm., and methemoglobin 7.4 Gm. per 100 ml. of blood prior to the beginning of therapy. Aspiration of sternal bone marrow yielded a moderately thick marrow which was also chocolate brown in color. The centrifuged marrow showed fat 5 per cent, plasma 12 per cent, buffy (myeloid erythroid) layer 9 per cent, and red cells 3 per cent. Marrow smears showed an erythroid hyperplasia at the normoblast level (Fig. 1).





wished to be discharged from the hospital. He was placed on outpatient status and given a supply of ascorbic acid but he failed to return for follow up. Two months later he returned for more ascorbic acid, and further data were then obtained. His skin color was definitely a normal pink hue, and the venous blood had a normal dark red color. The laboratory findings at this time are listed as '83rd day' in Table I and Fig 2. The bone marrow showed no significant change.

#### DISCUSSION

This case was of interest for several reasons: the chronicity of the illness, the high initial methemoglobin level and the response during the period of therapy. The patient was able to carry on strenuous work over a period of years as a merchant seaman without dyspnea, fatigue or other constitutional symptoms. Finch,<sup>1</sup> who reviewed this subject exhaustively in 1948, stated that he saw a patient with a methemoglobin level of 40 per cent who was able to carry on strenuous work. Our patient had 37.2 per cent. A more remarkable point of interest arose during the course of therapy. Although the usual response with treatment is a reduction in methemoglobin and total hemoglobin, our patient exhibited a different course. As the methemoglobin decreased the total hemoglobin remained stationary and the oxyhemoglobin level rose. The red cell count and hematocrit also remained fairly stationary. This was entirely contrary to our expectations since we anticipated hemolysis of the excess erythrocytes as the methemoglobin was reduced. Undoubtedly the chronic anoxemia present for so many years proved a potent stimulus to the erythrogenic elements of the bone marrow. Had we been able to follow the patient for a longer period it would have been interesting to note how long a time elapsed before the number of circulating red cells became reduced in number.

At least sixteen proved cases of congenital idiopathic methemoglobinemia had been reported in the literature up to the time of Finch's review (1948). In eight there was a familial incidence. In 1947, Coudounis and associates<sup>2</sup> reported fourteen cases all occurring in a Greek family within four consecutive generations, four pairs of siblings were affected by the syndrome. The name of the family was Vophtochlari which the authors translated as 'people with discolored lips'. Sievers and Ryon<sup>3</sup> stated that they were able to tabulate eighteen reported cases up to 1945 and they added one of their own. Gibson and Harrison (1947)<sup>4</sup> reported five cases in one family. Bensley, Rhea and Mills (1938)<sup>5</sup> reported two cases occurring in a brother and sister and stated that these were the first in which the diagnosis was established definitely. They added that the cyanosis was constant and was not affected by any form of therapy. Vitamin C was not used.

Other cases have been reported in the literature from time to time. In some, history of drug intoxication could not be ruled out. Others were given the name of "enterogenous cyanosis" supposedly associated with constipation and production of large amounts of nitrates by the bacteria of the intestinal tract.

To Lian<sup>6</sup> is ascribed the first use of ascorbic acid in the treatment of methemoglobinemia. It was believed to be superior to methylene blue. Ascorbic

acid has been used in many of the cases reported since then, almost all with excellent results<sup>10</sup> The oral route is as effective as the parenteral, and Graybiel and co-workers<sup>9</sup> have found that concomitant administration of sodium bicarbonate decreases the urinary excretion of vitamin C and the rate at which it disappears from the blood King, White, and Gilchrist<sup>10</sup> found that regardless of the amount of ascorbic acid given to their patient, the methemoglobin level could not be reduced below 7 per cent of the total blood pigment Gibson and Harrison<sup>6</sup> also found that they were not able to reduce the total methemoglobin below 10 Gm per 100 ml of blood by the use of vitamin C Our findings, over a short period of time, are similar to these

Normally, the mammalian anuclear erythrocyte reduces methemoglobin by an enzymatic process in which glucose and lactate are the principal substrates Clinical methemoglobinemia may then be produced by absence or failure of this reconversion mechanism (primary) or by the action of oxidants which produce methemoglobin more rapidly than the cell is able to reduce it (secondary) Further, the oxygen dissociation curve is normal in primary methemoglobinemia, whereas that induced by drugs shows a shift to the left<sup>1</sup>

Gibson proposes that the erythrocytes of primary methemoglobinemic patients are deficient in coenzyme factor I, which functions with those phosphoric and lactic enzymes of the red blood cell to facilitate reduction of methemoglobin to hemoglobin Methylene blue is therefore thought to catalyze reduction of methemoglobin by enzymes otherwise unable to bring about this reaction Ascorbic acid, by contrast, reduces methemoglobin directly, but is never complete<sup>6</sup>

These differential diagnostic features are important in providing the proper therapy for methemoglobinemia

#### SUMMARY

A case of congenital idiopathic methemoglobinemia in a 61-year old man is presented The patient was treated with daily oral doses of 300 mg of ascorbic acid and showed an excellent, though somewhat unusual, response to therapy The case is of interest because of the age of the patient, the chronicity of the disease, and the trend during therapy Some of the pertinent literature is reviewed briefly

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## LABORATORY METHODS

### A SIMPLIFIED TURBIDIMETRIC METHOD OF AUREOMYCIN ASSAY FOR CAPILLARY BLOOD AND OTHER BODY FLUIDS

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FROM studies on various methods of bio-assay for aureomycin, a turbidimetric method has evolved which is sufficiently accurate and sensitive in the presence of body fluids for investigative purposes and is sufficiently simple for routine use. It also meets a prime requirement for pediatric use in that an amount of blood adequate for serum assay can be obtained from a single finger or heel puncture. Osgood and Graham<sup>1</sup> developed a similar turbidimetric method of assay for penicillin, streptomycin, and trivalent organic arsenicals. Meads and co-workers<sup>2</sup> adapted it to the assay of aureomycin. We found that for aureomycin the accuracy of this method in the presence of body fluids is increased appreciably by substituting a standard curve containing four points for the single standard used by the original workers and that this change also simplifies the method by eliminating most of the mathematical details involved in computing the results.<sup>3</sup> Several other minor modifications were also helpful. It therefore seems worth while to publish the details of this method as it is being used in our laboratory.

#### DETAILS OF TECHNIQUE

**Materials**—A Klett Summerson colorimeter with a No. 660 filter, sterile matched colorimeter tubes with rubber stoppers, 0.85 per cent saline, 1 and 5 cc pipettes, a 37° C water bath, an ice water bath in the refrigerator, and standardized, uncontaminated test organism (Oregon J strain of *Staphylococcus aureus*—American Type culture collection No. 9801) are used.

The organism is standardized as follows. 5 ml amounts of a fresh one to three hour broth culture\* are added to 100 ml amounts of sterile Bacto Tryptose Phosphate Broth (Difco), which is then incubated in the 37° C water bath until the optical density of the culture is determined by colorimeter readings at fifteen minute intervals is approximately fifteen points (twelve to eighteen points) greater than that of a broth blank. This usually takes two and one half to three hours. At this point, growth is stopped by transferring the culture to the ice water bath. Thus, the standardized culture, is kept in the ice water bath until used and is made up fresh every four days.

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The aureomycin used in this study was supplied by Lederle Laboratories Division of the American Cyanamid Company.

These studies were conducted under contract with the Department of the Army Chemical Corps Camp Detrick Maryland.

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\*This is prepared by inoculating Bacto-Tryptose Phosphate Broth from the stock culture. The stock culture is kept on a nutrient agar slant in the refrigerator. A fresh slant is prepared every two weeks. Each new slant is checked for contamination before use.

A series of previously matched cotton plugged sterile colorimeter tubes is arranged for assay as shown in Table I. As soon as all dilutions are made, sterile rubber stoppers are inserted into the tubes and the solutions agitated by inversion of the tubes. The colorimeter is set at zero with the blank tube, and the turbidity of each remaining tube is read rapidly in a predetermined order. Immediately after reading each tube is placed in the 37° C water bath. Four hours later each tube is individually removed from the water bath, wiped, agitated, and reread in the same order.

TABLE I. MATERIALS ADDED TO TUBES FOR TURBIDIMETRIC ASSAY OF AUREOMYCIN IN BODY FLUIDS

TUBE	APPROPRIATE BODY FLUID (ML)	SALINE (ML)	MICROGRAMS OF AUREOMYCIN PER ML OF SALINE†		STANDARDIZED TEST CULTURE‡ (ML)
			FOR URINE ASSAY	FOR SERUM AND SPINAL FLUID ASSAY	
Blank	0	5.0	0	0	0
Control	0.5	0.5	0	0	4.0
Standard 1	0.5	0.5	2.0	1.0	4.0
2	0.5	0.5	1.0	0.5	4.0
3	0.5	0.5	0.5	0.25	4.0
4	0.5	0.5	0.25	0.125	4.0
Unknown 1	0.5	0.5	0	0	4.0
2	0.5	0.5	0	0	4.0
Etc	0.5	0.5	0	0	4.0

\*Contains no added aureomycin

†Aureomycin dilutions freshly made from a 5000 µg per milliliter of aureomycin solution which is made up fresh every two weeks from lyophilized aureomycin and kept at 4° C

‡Added last while still at 4° C

Computation of the results hinges on the principle demonstrated by Good† that in the case of a sensitive organism when sufficient bacterial growth has occurred to provide for differences that can be accurately measured the concentration of the appropriate antibiotic is proportional to the square of the difference between the turbidity of a control tube and the turbidity of a tube containing the antibiotic. The details are described as follows and are illustrated in Table II. The increase in turbidity between the four hour reading and the zero hour reading is determined in each of the four standard tubes (containing known amounts of aureomycin), each of the unknown tubes (containing the body fluid to be assayed) and the control tube (containing no aureomycin). The increase in turbidity (which is the measurement of bacterial growth during the four hours of incubation) of each standard tube and each unknown tube is then subtracted from the increase in turbidity of the control tube. The difference in turbidity increase between the control

TABLE II. COMPUTATION OF RESULTS FOR A SERUM STANDARD CURVE

TUBE	AUREOMYCIN (γ PER ML)	TURBIDITY IN KLETT UNITS		DIFFERENCE IN TURBIDITY INCREASE BETWEEN CONTROL AND STANDARD	DIFFERENCE IN TURBIDITY INCREASE BETWEEN CONTROL AND STANDARD SQUARED
		0 HR †	4 HR		
Control	0	46	133	87	
Standard 1	1.0	47	70	23	64
2	0.5	47	85	8	49
3	0.25	44	102	58	34
4	0.125	46	121	75	144

Concentration in saline added to tubes. Final concentration is one tenth of this

†This reading corrects for slight differences in optical density of the individual tubes and individual specimens

tube and each of the standard and unknown tubes is squared and is, by definition, proportional to the amount of antibiotic present. The squared values for the standards are plotted along the abscissa of semilog paper against the known concentrations of aureomycin per milliliter of saline (see Fig 1 and Table I). A standard curve is drawn through the four points obtained. The concentration of aureomycin in the unknowns is read off this curve using the squared values for the unknowns obtained as described.

#### SERUM STANDARD CURVE

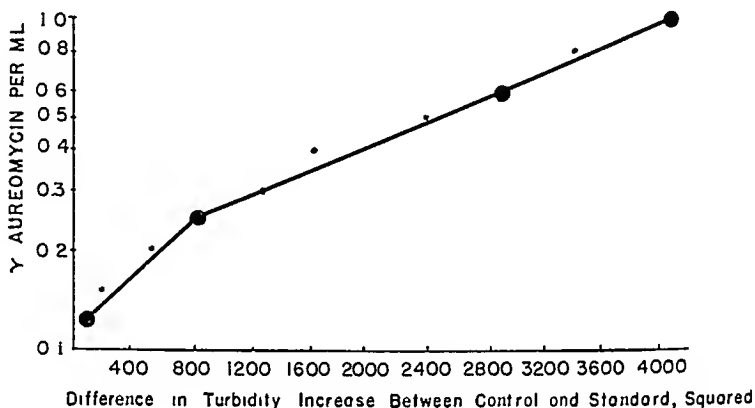


Fig 1—The four standard points are plotted in large dots while twelve simultaneous intermediate values are plotted in small dots. The concentrations of aureomycin plotted on the ordinate of this graph are ten times the final concentration of aureomycin in the standard tubes and therefore correspond to the concentration of aureomycin in an unknown solution before it is diluted by addition of saline and standardized test culture (see Table I).

Blood is collected from a finger or heel puncture and the serum is separated for assay as previously described by one of us (A D H).<sup>4</sup> The collecting tube is made either from a three inch length of glass tubing, with one end sealed, or from a 75 by 95 mm shell vial. The open end is drawn out to a capillary tip and sealed. The wide portion of the tube is held over the flame and an air vent is quickly produced in the softened glass by the expanding air within the sealed tube. For the collection of blood, the capillary tip is broken off near its base. The finger or heel is punctured so that a free flow of blood is obtained. The tube is held in as nearly vertical a position as possible and the blood readily flows into the tube and down to the base. The tubes are stood upright until the blood is clotted, then the tube is filed off just above the surface of the blood. The clot is broken up with an applicator stick and the tube is centrifuged at 1,500 to 2,000 revolutions per minute for fifteen minutes. The supernatant serum is then pipetted off with a capillary pipette for assay. After a little practice with this technique, one can consistently collect 15 ml or more of capillary blood from a single skin puncture.

Unknown serum and spinal fluid specimens are run in 1:1 and 1:8 dilutions,\* thus bringing a maximum of 8 μg per milliliter of aureomycin within the detectable range of the method. Similarly, unknown urine specimens are run in 1:20 and 1:160 dilutions to detect a range of 5 to 320 μg per milliliter of aureomycin. These predilutions are made in the type of body fluid being assayed. Serum† for them is obtained from the blood bank,

\*For maximum accuracy a separate pipette is used for each step in the dilution of serum specimens.

†Twenty individual specimens of human serum were tested simultaneously under the conditions of this method and no significant difference was found in the growth of the test organism among these sera. Serum however does stimulate the growth of the test organism markedly when compared with the assay broth. Keeping the serum concentration constant in all the tubes of a serum assay controls this factor. However the concentration of aureomycin in an unknown specimen is calculated from the tube with the greatest predilution when there is a choice since most of the body fluid in prediluted specimens comes from the same source as the body fluid used in the standard and control tubes.

spinal fluid from air encephalographic studies and urine from laboratory personnel. All body fluid for dilution of unknown specimens is kept frozen until used. Correction for such dilutions is made in calculating the results.

Aureomycin in a concentration of 1  $\mu\text{g}$  per milliliter in serum and 40  $\mu\text{g}$  per milliliter in acid urine shows no appreciable loss of activity when kept at  $-20^\circ\text{C}$  for one week. However, aureomycin in spinal fluid in a concentration of 0.25  $\mu\text{g}$  per milliliter shows about a 50 per cent loss of activity if kept at  $-20^\circ\text{C}$  overnight. Therefore unknown specimens of serum and acid urine are kept at  $-20^\circ\text{C}$  until a convenient time for assay, but spinal fluid unknown specimens are assayed on the day collected. The pH of all urine used is adjusted to 7.2 just before assay.

Replicate determinations of either control standards, or unknowns do not appreciably enhance the accuracy of the method. A separate standard curve is required for each type of body fluid on each day of assay.

### RESULTS

The results of one hundred and four consecutive determinations of known concentrations of aureomycin intermediately spaced between the values used to make up the standard curve are tabulated in Tables III, IV, and V. One experiment using serum is illustrated in Fig. 1 in which it will be noticed that the standard curve approaches a straight line on semilog paper. A similar straight line is obtained with the other two body fluids tested. The percentage error of each determination has been computed. For urine, the range of error is 0 to 42 per cent with a mean of 12.4 per cent, for spinal fluid the range is 0 to 35 per cent with a mean of 12.3 per cent, for serum the range

TABLE III RECOVERY OF KNOWN CONCENTRATIONS OF AUREOMYCIN IN URINE FROM 36 CONSECUTIVE DETERMINATIONS

KNOWN $\gamma$ AUREOMYCIN PER ML.	1.60	1.30	0.90	0.60	0.40	0.30
Recovered $\gamma$ aureomycin per ml						
Determination 1	1.48	1.20	0.84	0.50	0.36	0.26
2	1.48	1.33	0.84	0.60	0.39	0.27
3	1.50	1.33	0.86	0.62	0.50	0.33
4	1.55	1.40	0.90	0.62	0.50	0.36
5	1.55	1.40	1.00	0.7	0.50	0.38
6	1.73	1.55	1.00	0.7	0.50	0.45

TABLE IV RECOVERY OF KNOWN CONCENTRATIONS OF AUREOMYCIN IN SERUM FROM 36 CONSECUTIVE DETERMINATIONS

KNOWN $\gamma$ AUREOMYCIN PER ML.	0.80	0.60	0.40	0.30	0.20	0.15
Recovered $\gamma$ aureomycin per ml						
Determination 1	0.60	0.56	0.35	0.26	0.14	0.13
2	0.74	0.60	0.36	0.28	0.16	0.13
3	0.77	0.60	0.39	0.29	0.15	0.14
4	0.78	0.62	0.41	0.31	0.19	0.14
5	0.78	0.62	0.42	0.31	0.20	0.14
6	0.92	0.71	0.45	0.35	0.20	0.15

TABLE V RECOVERY OF KNOWN CONCENTRATIONS OF AUREOMYCIN IN SPINAL FLUID FROM 32 CONSECUTIVE DETERMINATIONS

KNOWN $\gamma$ AUREOMYCIN PER ML		0.87	0.80	0.75	0.62	0.60	0.40	0.37	0.30	0.20	0.15
Recovered $\gamma$ aureomycin per ml											
Determina tion	1	0.67	0.73	0.47	0.53	0.57	0.33	0.29	0.25	0.17	0.13
	2	1.00	0.78	0.47	0.53	0.62	0.33	0.29	0.26	0.19	0.13
	3	—	0.78	—	—	0.62	0.40	—	0.30	0.20	0.14
	4	—	0.78	—	—	0.68	0.44	—	0.32	0.20	0.14

is 0 to 35 per cent, with a mean of 83 per cent. The accuracy of this method appears to be distinctly superior to that we achieved, in the presence of body fluids, with the original turbidimetric method of Meads and a serial dilution method of aureomycin assay.<sup>3</sup> Furthermore, it is possible to detect 0.1  $\mu\text{g}$  of aureomycin per milliliter or less in the presence of the three body fluids tested, so that its sensitivity compares favorably with the sensitivity of the other methods currently in use.<sup>3</sup> The method is not as accurate in concentrations below 0.125  $\mu\text{g}$  per milliliter as in higher concentrations, however.

#### CONCLUSION

A modification of Meads' turbidimetric method of aureomycin assay has been described. It appears to provide a simple and accurate means of assaying low concentrations of aureomycin in small volumes of various body fluids.

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## A MICROMETHOD FOR BLOOD PENICILLIN ASSAY

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IN MAKING frequent penicillin plasma determinations in children, it is helpful if a method can be used which requires such a small amount of blood for each assay that it can be obtained from a puncture wound of the finger as opposed to withdrawal by venipuncture. Fielding<sup>1</sup> described a micromethod requiring as little as 0.25 ml. of fluid for assay in which the test organism was the Oxford strain of *Staphylococcus aureus* grown in glucose serum medium containing Andrade's indicator. The principle of the test was the detection by means of the color indicator, of the pH change induced in the medium by the breakdown of glucose during the growth of the test organism. When the growth of the organism was prevented by a given amount of penicillin then no color change would occur. The minimum amount of penicillin which prevented the growth of the organism was that present in the last tube showing no color change.

In the procedure described by Fielding, the test organism and body fluid to be tested were mixed on a paraffined slide and then taken up in capillary tubes following the technique of Fleming<sup>2</sup>. Because this technique is not commonly employed routinely in this country it seemed advisable to attempt to utilize the same principle and devise a micromethod employing the more conventional procedures involved in serial test tube dilutions. The method to be described fulfills the following criteria: (1) It requires only that amount of blood which can be taken easily from a finger prick. (2) It uses an easily prepared test medium. (3) The end point is easily read by means of a sharp color change. (4) The procedure is one commonly employed in serology. (5) Finally its accuracy appears to be superior to that obtained by the widely employed method described originally by Rammelkamp<sup>3</sup> and frequently modified.

### TECHNIQUE

*Collecting of Blood*—The tube which was found convenient for drawing blood and separating the serum has been described previously<sup>4</sup> and is illustrated in Fig. 1\*.

Blood was taken from finger or heel puncture by capillary attraction and allowed to clot in the body of the tube. The tube was then filed and broken off above the level of the blood which was then centrifuged at 1500 to 2000 rpm for ten to twenty minutes. The serum that separated was pipetted off for testing.

*Organism*—The organism used in this laboratory was *Staph. aureus* (F.D.A. strain 909). There is no reason why any penicillin sensitive easily cultivated organism could not

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Shell vials 9 by 7.5 mm. or glass tubing of 7 mm. bore and of convenient length (90 to 100 mm.) sealed at one end can be used. The open end is drawn out to capillary tip and sealed. A small hole is blown through the wall of the tube by heating it 1 mm. below the beginning of the capillary portion.

be used equally well. The organism, after a preliminary incubation at 37° C, was kept as a stock culture on plain nutrient agar (Difco) in a refrigerator. It was subcultured once a week onto fresh agar. When required for the test, the organism was transferred to nutrient broth (Difco) and incubated at 37° C for eighteen hours. A portion was then diluted 1:1,000 in the assay medium to be described below.

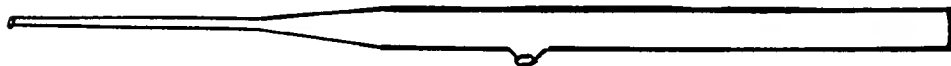


Fig. 1

*Medium*—The assay medium was phenol red dextrose broth made up as follows:

Nutrient broth (Difco)	8 Gm
Dextrose	5 Gm
Aqueous phenol red	25 ml
Distilled water	1,000 ml

This was adjusted to pH 8 with N sodium hydroxide using the phenol red (0.04 per cent concentration) as indicator, the final color being red. After autoclaving for fifteen minutes at 15 lb, the medium was ready to use without further adjustment of the pH.

*Procedure*—The test was arranged so that one half step dilutions were used, and a triplicate penicillin control test was included with each series of unknowns.

*Control*—This was included routinely to detect the sensitivity of the test organism. A penicillin solution was made up in the assay broth in such a way that the penicillin concentration was 0.5 unit per milliliter. The one half step dilutions were made as follows: Three sets, each containing thirteen plugged, sterile Wassermann tubes (100 by 13 mm), were arranged in suitable racks. In each set of tubes, the following steps were carried out. The test medium (phenol red dextrose broth) was added in 0.1 ml amounts to tubes 1, 3, and 4 to 12 inclusive. Tube 2 was left empty, and 0.2 ml of the test medium was added to tube 13. The penicillin solution was then added in 0.1 ml amounts to tube 1 which made a 1:2 dilution and to tube 13 which made a 1:3 dilution. Tube 1 (1:2 dilution) was then used after thorough mixing, for preparing one series of twofold (one step) dilutions by transferring 0.1 ml, serially to the odd numbered tubes 3 to 11, 0.1 ml from tube 11 being discarded. From tube 13, which contained 1:3 dilution of penicillin, 0.1 ml was added to the empty tube 2 (1:3 dilution) and to tube 4 which already contained 0.1 ml assay broth (1:6 dilution). Tube 13 was then discarded. Tube 4, after thorough mixing, was used to initiate the second series of twofold (one step) dilutions by transferring 0.1 ml serially to the even numbered tubes 6 to 12, 0.1 ml from tube 12 being discarded. Tubes 1 to 12 in each set then contain 0.1 ml of assay medium, with concentrations of penicillin decreasing from 1:2 to 1:96 by one half steps.

*Test Fluids*—A maximum of 0.2 ml was required for each test fluid. Except that only one set of tubes was run, this was handled in a similar manner to that just described, the fluid taking the place of the known penicillin solution.

*Test Organism*—0.1 ml of the 1:1,000 dilution of the eighteen hour culture of the test organism described was then added to every tube and the complete series incubated overnight at 37° C.

*End Point*—Since, with this indicator, a change from red to yellow indicates a lowering of pH resulting from bacterial growth, the end point was taken as the last tube in each set in which the original red color of the medium was unaltered. This end point was usually quite sharp. Since the controls were set up in triplicate, it sometimes occurred that the end point varied somewhat in one of the three sets, although this variation was seldom more than one half step off in either direction. The end point taken was that reached in two of the three control sets. If there was no such agreement, the test was considered invalid.

**Calculation**—First, the sensitivity of the organism was calculated from the controls. Since the concentration of penicillin in the first tube of each control series was 0.5 unit per milliliter, the concentration in any subsequent tube could be calculated by dividing 0.5 unit per milliliter by the reciprocal of the dilution of that tube. For example, if the end point in two of the three control series was found to be tube 7 or a 1:16 dilution, then the organism was sensitive to a concentration of  $\frac{0.5}{16}$  or 0.03 unit per milliliter.

The concentration of penicillin in any unknown body fluid was easily calculated by multiplying the sensitivity of the organism, as determined in the preceding paragraph, by the reciprocal of the dilution of body fluid contained in the end point tube. For example, if in the test the sensitivity of the organism was 0.03 unit per milliliter and the end point of the body fluid being tested was found to be tube 5 or a 1:8 dilution, then the concentration of penicillin in the original body fluid was  $0.03 \times 8$  or 0.24 unit per milliliter.

In the laboratory it was found convenient to record the dilution and the end points of the controls and the test fluids on squared paper. This avoided any mistake in the dilutions (Table I).

TABLE I. SAMPLE CHART FOR MICRO PENICILLIN ASSAY

TUBE	1	2	3	4	5	6	7	8	9	10	11	12	PENICILLIN (UNITS/ML.) DETECTED
DILUTION	—	3	4	6	8	12	16	24	32	48	64	96	
PENICILLIN (UNITS/ML.)	0.25	0.17	0.125	0.085	0.065	0.04	0.03	0.02	0.015	0.01	0.0075	0.005	
Control 1							x						} 0.03
2							x						
Pt 1	1/2 hr						x						0.06
1 1/2 hr							x						0.06
3 hr													0.12
Pt 2	1/2 hr												1.44
1 1/2 hr								x					0.48
3 hr													0.18
Pt 3	1/2 hr												0.48
1 1/2 hr													0.48
3 hr													0.24

**Accuracy of the Method**—This method was first tested on five different occasions by attempting on each occasion to determine the amount of penicillin in each of five twofold serial dilutions of a known concentration of penicillin in distilled water. Table II shows the results obtained by the method just described. It can be seen that the recoveries at each dilution were remarkably close to the amount of penicillin actually present. The average of the tests was  $\pm 8$  per cent, although individual readings sometimes deviated as much as 44 per cent. Comparison with the results obtained by the Rammelkamp method

TABLE II. DETERMINATION OF ACCURACY OF MICROMETHOD OF PENICILLIN ASSAY

KNOWN PENICILLIN CONCENTRATION	UNITS/ML. OF PENICILLIN IN SERUM				
	0.1	0.5	1.0	2.0	4.0
Test 1	0.12	0.48	1.08	1.92	3.84
Test 2	0.12	0.72	1.08	1.92	3.84
Test 3	0.12	0.48	1.08	1.92	3.84
Test 4	0.12	0.72	0.72	1.92	84
Test 5	0.09	0.48	1.08	1.92	3.84
Average	0.11	0.57	1.0	1.92	84

(Table III) indicates that the micromethod was more accurate. However, there appeared to be a definite relationship between the two, the micromethod giving a consistently higher reading by a factor of 1.6. Fig. 2 shows a graphic comparison of the known concentrations of penicillin and the recoveries by the two methods used.

TABLE III DETERMINATION OF ACCURACY OF THE MODIFIED RAMMELKAMP METHOD OF PENICILLIN ASSAY

KNOWN PENICILLIN CONCENTRATION	UNITS/ML OF PENICILLIN IN SERUM				
	0.1	0.5	1.0	2.0	4.0
Test 1	0.052	0.3	0.832	1.22	2.43
Test 2	0.052	0.3	0.832	1.22	2.43
Test 3	0.052	0.3	0.624	1.22	2.43
Test 4	0.052	0.3	0.624	1.22	2.43
Test 5	0.052	0.3	0.624	0.91	2.43
Average	0.052	0.3	0.71	1.15	2.43

COMPARISON OF MODIFIED FIELDING AND RAMMELKAMP METHODS OF PENICILLIN BIO-ASSAY

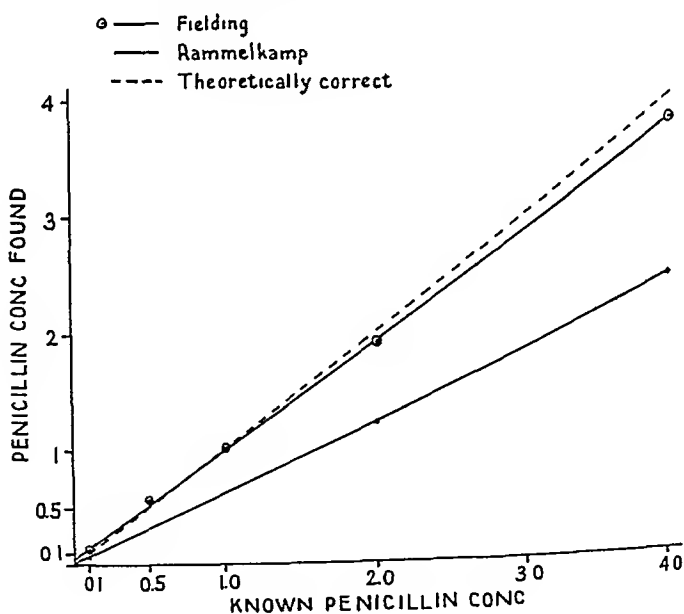


Fig. 2

Following the demonstration that known amounts could be assayed with a good degree of accuracy, this test has been used successfully for hundreds of routine assays of penicillin levels in body fluids.

#### SUMMARY

A micromethod for the estimation of penicillin levels in blood or other body fluids, requiring only 0.2 ml for the test, is described. Sufficient blood can be collected conveniently from a single puncture wound of the finger. A convenient collecting tube for obtaining serum is described and illustrated.

The principle employed in the test is the detection of growth of the test organism in a glucose containing medium by the demonstration of acid formation by the color change of a suitable indicator. The end point is taken as the last tube to show no evidence of color change. By including controls in each test, the quantity of penicillin in an unknown penicillin fluid can be calculated. The accuracy of the method is greater in our hands than the commonly employed Rammelkamp method and it has proved a convenient assay method for routine use in children.

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# THE PRODUCTION OF ANTIRABBIT HEMOLYSIN

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THE hemolytic system most commonly used in complement fixation tests consists of sheep erythrocytes and antish sheep hemolysin. A supply of fresh sheep erythrocytes for the performance of the test necessitates constant maintenance of a few sheep on the premises, which is often inconvenient. Attempts have been made by Vedder<sup>1</sup> and by me<sup>2</sup> to overcome this difficulty by a different hemolytic system. The object of the present communication is to describe another modification, in which the role of rabbit and sheep is reversed. Rabbit erythrocytes are employed with an antirabbit hemolysin of the sheep.

## METHODS AND MATERIALS

Five sheep were selected for this study, two adult males, two adult females, and one female lamb.

To each 50 ml of freshly drawn rabbit blood about 2 ml of 0.4 per cent sodium citrate were added to prevent clotting. The erythrocytes were washed three times in sterile 0.9 per cent NaCl solution and the packed red blood corpuscles were resuspended in equal volume of saline for inoculum.

Hemolyzed rabbit red blood corpuscles and the stroma of rabbit erythrocytes as prepared by Vedder were tried out as an alternate inoculum.

The inoculations were either intravenous or subcutaneous or both, and the dose for each inoculum varied from 5 ml to 200 milliliters. Control blood samples were taken from the sheep before initial inoculations. Samples were taken also at the end of each course of immunization and after inactivation at 56° C for thirty minutes they were tested for hemolysin.

Antirabbit hemolysin titrations were made with 2 per cent washed rabbit erythrocytes. In some of the tests, titrations were made with blood taken from the same rabbits that had been bled previously for immunization of the sheep.

Pooled guinea pig complement in dilutions of 1:15 and 1:30 was used in the hemolysin titrations. The titration was checked by using the standard Kolmer hemolysin titration test with antish sheep hemolysin and 2 per cent sheep red corpuscles.

Wassermann positive and Wassermann negative human sera were tested with the antirabbit hemolysin and 2 per cent washed rabbit erythrocytes by the modified Kolmer method and with the standard Kolmer Wassermann complement fixation test, Kolmer,<sup>3</sup> and the results were compared.

## EXPERIMENTAL DATA

The five sheep used in this study differed in their production of antirabbit hemolysin.

The sheep which were inoculated with large doses of rabbit erythrocytes at frequent intervals produced hemolysin promptly and in higher titer as compared with the sheep which were inoculated with smaller doses of erythrocytes.

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even when inoculations were spread over a longer period of time. When sheep were immunized for hemolysin, it was noted that agglutinin for rabbit erythrocytes occurred much sooner and in higher titer than did antirabbit hemolysin, particularly when the period of immunization was prolonged. Thus Sheep A at the end of seventeen days had no hemolysin while the agglutinin titer was 1:2,400. The same sheep at the end of ninety-three days of immunization had a hemolysin titer of 1:30 and the agglutinin titer was 1:10,240.

In Sheep D and L, which were immunized in massive doses and at frequent intervals, agglutinin and hemolysin occurrence were almost simultaneous.

The results of the production of antirabbit hemolysin in sheep are summarized in Table I.

Sheep A (male) which was immunized with small quantities (5 to 10 ml) intravenously of washed rabbit erythrocytes produced hemolysin after sixty-nine days of immunization; however, during the entire course of immunization (ninety-three days) hemolysin titer never exceeded 1:30.

Sheep B (male) received sixty-nine injections totaling 250 ml of lysed rabbit erythrocytes and 2,455 ml of washed rabbit erythrocytes in a period of 290 days. The immunization of this sheep was begun with small amounts (10 ml) of hemolyzed red blood corpuscles. During fifty days of immunization, this sheep was inoculated nineteen times with 190 ml of hemolyzed erythrocytes. At the end of this period, the hemolysin titer was only 1:4. Since the lysed corpuscles failed to produce a significant hemolysin titer and inasmuch as Sheep A already had produced hemolysin following injection of intact erythrocytes, at this stage the inoculation of lysed erythrocytes was discontinued. Subsequent inoculations with rather large doses (25 to 100 ml per inoculum) of intact erythrocytes increased the hemolysin titer to 1:80. Prolongation of immunization instead of increasing decreased the hemolysin titer to 1:20 (see Table I).

Sheep C (female) received thirty injections totaling 1,400 ml of washed rabbit erythrocytes and 110 ml of stroma of rabbit erythrocytes during a period of 234 days. The immunization of this sheep was begun with rather large doses of washed rabbit erythrocytes and the highest hemolysin titer obtained was 1:20. Since the sheep manifested mild anaphylactic symptoms, intravenous inoculations were substituted with stroma. In spite of further immunization with stroma, which subsequently was followed with intact erythrocytes, the hemolysin titer did not increase (see Table I).

Sheep D (pregnant female) was immunized with fifty-four injections totaling 4,200 ml of washed rabbit erythrocytes in a 212-day period. The immunization of this sheep was done with massive doses and at frequent intervals. Hemolysin with titer of 1:40 was present as early as twenty-three days following initial inoculation. While the sheep was undergoing immunization, it gave birth to a lamb. Four days after the lamb was born, blood samples taken from the sheep showed hemolysin titer of 1:320. Subsequent inoculations with 50 and 100 ml of washed erythrocytes were at times accompanied with slight to moderate anaphylactic reactions such as sneezing, coughing, discharge of feces and bloody urine, weakness, inability to move, salivation, tremors, and dyspnea.

TABLE I THE PRODUCTION OF ANTIRABBIT HEMOLYSIN IN SHEEP

SHEEP	TRA THROCYTES		III MOIYZED PLA THROCYTES		STOMACH		ROUTE OF INOCULATION	DAYS BETWEEN LAGH INOCULA TION	FIST PERIOD (DAYS)	SEROLOGIO TESTS	
	ML PER EACH INOCULA TION	TOTAL ML	ML PER EACH INOCULA TION	TOTAL ML	ML PER EACH INOCULA TION	TOTAL ML				DAYS AFTER FIRST INOCULA TION	HEMOLY SIN TITER
A	5	45	-	-	-	-	IV	2 to 3	26	17	0
	10	90	-	-	-	-	IV	2 to 8	3	69	1 24
	10	90	-	-	-	-	SC	1 to 5	3	93	1 30
	-	-	10	100	-	-	IV	2 to 4	3	26	0
B	-	-	10	90	-	-	SC	2 to 5	3	50	1 4
	-	-	5 40	60	-	-	IV and SC	3	4	---	---
	25	175	-	-	-	-	SC	4 to 5	5	96	1 40
	50	550	-	-	-	-	IV	3 to 6	2	140	1 80
	50	300	-	-	-	-	IV	1 to 3	2	---	---
	50	300	-	-	-	-	SC	Drily	4	161	1 80
	40 70	350	-	-	-	-	IV	2 to 3	20	218	1 40
	100	600	-	-	-	-	IV	2 to 3	3	239	1 20
	50	150	-	-	-	-	IV	1 to 3	26	290	1 20
	50	350	-	-	-	-	IV	3 to 4	2	23	1 10
C	50 100	600	-	-	-	-	IV	2 to 4	146	44	1 20
	-	-	5 8	70	-	-	IV	2 to 11	4	---	---
	50	450	-	-	-	-	SC and IV	1 to 2	30	231	1 10
	50	500	-	-	-	-	IV	2 to 3	2	23	1 40
D	50 100	700	-	-	-	-	IV	2 to 3	21	41	1 320
	50 100	700	-	-	-	-	IV and SC	2 to 3	3	77	1 80
	200	600	-	-	-	-	IV and SC	2	24	86	1 80
	100	700	-	-	-	-	IV	2 to 3	24	122	1 40
	50	150	-	-	-	-	IV	1 to 3	30	173	1 40
	50	600	-	-	-	-	IV	2 to 3	4	204	1 40
	50 100	250	-	-	-	-	IV and SC	1 to 3	---	212	---
	100 200	800	-	-	-	-	IV	2 to 3	24	17	1 10
	50	150	-	-	-	-	IV	1 to 3	30	68	1 20
	50	300	-	-	-	-	IV	2 to 3	2	85	1 10
L	50	300	-	-	-	-	IV	2 to 3	4	99	1 10
	50 100	750	-	-	-	-	SC and IV	1 to 12	30	137	1 20
	30 30	210	-	-	-	-	SC	1 to 12	12	190	1 10
	-	-	-	-	-	-	SC	1 to 12	12	190	1 10



In spite of continuation of immunization the hemolysin titer never exceeded 1:320, but diminished to 1:40. During the last intravenous inoculation, the sheep died of anaphylactic shock.

**Sheep D.** This female lamb born to immunized Sheep D was tested at the age of 2 months for the presence of possible anti-rabbit hemolysin with negative results. This was followed by forty injections of rabbit red blood corpuscles totaling 2510 ml during 180 days. The first seven inoculations were made intravenously in 200 ml amounts every other day. The highest hemolysin titer obtained at various stages of immunization was 1:20 (see Table I).

Anti-rabbit hemolysin with a titer of 1:40 or above and a 2 per cent suspension of washed rabbit erythrocytes was tested with Wassermann positive and Wassermann negative human sera by Kolmer technique because of the low hemolytic titer of anti-rabbit hemolysin. Relatively large amounts of this hemolysin were used. As a control parallel tests were made with Kolmer Wassermann method. Forty two syphilitic and forty two normal sera were run by each method. The results of this test were clear cut and known positive samples gave positive readings and negative samples gave negative readings by both procedures. The findings by the experimental method were identical with those obtained with anti-sheep hemolysin and washed sheep erythrocytes as used in the usual Wassermann test.

#### SUMMARY

Anti-rabbit hemolysin was produced in sheep by inoculating them intravenously and subcutaneously with washed rabbit red blood corpuscles. The hemolysin titer required by these animals ranged from 1:10 to as high as 1:320.

Hemolyzed erythrocytes and stroma injected into sheep for the production of hemolysin did not produce as high a titer as washed erythrocytes.

Anti-rabbit hemolysin and 2 per cent washed rabbit erythrocytes were used in testing syphilitic and normal sera by modified Kolmer Wassermann technique. The results obtained in every instance were clear cut and of diagnostic value.

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# DIFFERENTIATION AND ENUMERATION OF EOSINOPHILS IN THE COUNTING CHAMBER WITH A GLYCOL STAIN, A VALUABLE TECHNIQUE IN APPRAISING ACTH DOSAGE

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THE decrease in the circulating eosinophils following the intramuscular injection of adrenocorticotrophic hormone (ACTH, Airmou) was first described by Hills, Forsham, and Finch<sup>1</sup> and subsequently by Hellman.<sup>2</sup> The increasing importance of ACTH in the diagnosis<sup>3</sup> and treatment<sup>4, 5</sup> of various clinical conditions as well as in other clinical studies of the effects of this hormone<sup>6, 7</sup> underscores the importance of a simple, reliable technique for enumerating eosinophils, as the level of these cells in the peripheral blood may be a general index of the sensitivity of the gland stimulation and may be a general guide to adequacy of dosage in a given case.<sup>8, 9</sup>

In this connection the merits of a white blood cell diluting fluid of equal parts propylene glycol and water containing phloxine and methylene blue as stains should be re-emphasized because it is the simplest and most accurate method of enumerating blood eosinophils. This point is important because the number of circulating eosinophils is a general guide of adequate dosage of ACTH in treating various disease syndromes. Although our own observations in this respect† are not ready for publication, a review of the technique applied in enumerating eosinophils seems indicated because of the rapidly increasing medical interest in adrenocorticotrophic hormone (ACTH).

Hypotonic diluting fluids of the type originally described by Dungei<sup>10</sup> or as modified by Hills, Forsham, and Finch<sup>1</sup> and Thoin and associates<sup>3</sup> are not entirely satisfactory for counting eosinophils in the peripheral blood. This statement is based on the experience of employing Camaia and Alvarez<sup>11</sup> modification of Dungei's diluting fluid consisting of 5 parts of 1 per cent aqueous eosin, 5 parts acetone, and 100 parts distilled water for a period of three years in studying cases of food and drug allergy. The staining fluid used by those studying the effects of ACTH is identical except that 2 per cent instead of 1 per cent aqueous eosin was employed.

The inadequacies of this type of diluting fluid were first pointed out in 1944.<sup>12</sup> It was determined that the counting chamber estimation of eosinophils by means of hypotonic diluting fluids containing eosin as modified from Dungei's original description remains subject to considerable error in that the ruptured or "ghost" cells are identified by clumps of eosin-staining granules retained in portions of the cell membrane. In 1947 it was again emphasized by me<sup>13</sup> that the use of such diluents was fraught with considerable error in that one frequently encountered indeterminate forms that could not with certainty

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†These studies were conducted through the cooperation of Dr David E. Markson and Dr Smith Freeman who are studying the effect of ACTH on disease syndromes. The ACTH was obtained through the courtesy of Dr John R. Mote of the Armour Laboratories.

be classified as eosinophils. In addition, a greater absolute number of eosinophils usually was obtained from employing this type of hypotonic diluent than was determined from performing parallel stained film differential counts. It was further pointed out, however, that the errors inherent in this method became less significant if the determinations were made with a short but constant time interval elapsing between the dilution of the sample and the completion of the counting. These observations have recently been confirmed by Henneman, Wexler, and Westenhaver<sup>14</sup>. In their experience with the Dinger technique as modified by Thorn and associates there was a large and rapid decrease in the eosinophil cell count with passage of time after dilution of the blood and with ageing of the oxalated blood. Because of this and other reasons, these authors were unable to recommend the use of this type of diluent.

The so called "hemolytic" and solvent properties of propylene glycol suggested its use as a white cell diluting fluid and staining base in 1943<sup>15</sup>. Upon closer observation it was learned that the apparent hemolytic action of the glycols, as reported by Von Oettingen and Jirouch<sup>16</sup> was not a true hemolysis, but that the disappearance of human erythrocytes suspended in equal parts propylene glycol and water was due to the fact that the red blood cells rapidly assumed the same density as the surrounding media and thus became relatively nonrefractile. This phenomenon was studied in more detail by Randolph and Mallery<sup>17</sup> who were able to demonstrate that a mixture of human blood in equal parts propylene glycol and water appearing to be hemolyzed as viewed grossly in a test tube or under the light microscopic field actually was not hemolyzed when viewed through the dark field microscope. Under these circumstances the erythrocytes retained their normal contours.

The technique described in 1944<sup>12</sup> and employed in subsequent studies consists of the following:

Given amounts of stock solutions containing 0.1 per cent methylene blue in propylene glycol and 0.1 per cent phloxine in propylene glycol are each diluted with an equal volume of distilled water and placed in dropper bottles. For example:

Solution I*		
0.1 per cent methylene blue in propylene glycol		50.0 cc
Distilled water		50.0 cc
Solution II		
0.1 per cent phloxine in propylene glycol		50.0 cc
Distilled water		50.0 cc

The final white blood cell diluting fluid is made by mixing an equal number of drops of Solution I and Solution II in a test tube. This remains usable for approximately four hours. After standing for longer periods differential staining detail may become impaired and precipitation of dyes may occur. Curiously, prior to four hours the two dyes do not seem to mix completely as shown by the ability of the phloxine to disperse in a filter paper at a greater rate of speed than does the methylene blue. A drop on such a medium separating as to color and leaving a blue center and a red periphery in a manner akin to the principle employed in paper chromatography.

\* It has been pointed out the effect of propylene glycol on erythrocytes as far as the timing of the reappearance phase is concerned is dependent upon concentration of the glycol and the temperature. Occasionally the loss of water from the stock or working solutions due to evaporation increases the relative concentration of propylene glycol to the point that in summer weather the red cells may "reappear" before one has an opportunity to enumerate the leucocytes. This is remedied by making up solutions containing 40 or 45 per cent propylene glycol instead of the recommended 50 per cent.

Because of the variation in staining ability between lots of dyes, optimum acid and basic staining may sometimes be obtained by making a slight change in the relative proportions of Solution I and II in the final diluent. For example, four parts of Solution I and six parts of Solution II might result in better staining technique than if equal parts of the two were mixed.

Under certain circumstances the phloxine tends to crystallize out in long narrow crystals from Solution II, this may occur after the solution has stood several weeks and may be remedied readily by simple filtration or by preparing Solution II from the stock at monthly intervals.

Experience indicates that it is helpful to clean the pipettes with agents other than acids or to be certain that acid radicals are completely removed from the interiors of the pipettes as a result of repeated rinsing. Cleaning the pipettes with water and drying by a final rinse with acetone and avoiding the use of a series of bottles also employed in cleaning pipettes containing acid diluents aids in avoiding complications in the use of this technique.

Total and differential white blood cell counts are made using the standard pipette and counting chamber. The pipette is shaken during the course of and immediately after filling. If the chamber is charged immediately after diluting the blood and shaking, a period of from three to five minutes is required for the "disappearance" of the red blood cells and settling of the leucocytes prior to determining the total white cell count. A total period of from ten to fifteen minutes is recommended for maximum staining of eosinophils and other leucocytes. Blood may be left in the pipette for varying periods, including over night, without a significant change in the staining qualities of the cells or in the total leucocyte count, an obvious advantage over the use of hypotonic diluents employed in enumerating eosinophils.

Under the low magnification (16 mm objective) eosinophils may be differentiated from other cells by their brilliant red color and slightly larger size. The contrast in color is intensified by increasing the light and raising the condenser of the microscope. The eosinophils in one side of the counting chamber (0.9 cmm) or in the entire ruled areas of both sides of the chamber (1.8 cmm) are counted and multiplied, respectively, by 22 and 11 to obtain the number per cubic millimeter of blood.

The differentiation and enumeration of polymorphonuclear and mononuclear cells are accomplished to best advantage by the use of high magnification (4 mm objective and 15x eyepiece). The first 100 cells encountered are so classified.

In this diluting fluid all white blood cells remain intact, round in contour, and relatively large in size. The granules of the eosinophils stain brilliant red, the nuclei of all cells aquamarine, this permits the differentiation of eosinophils from other white blood cells even in the presence of highly fragile leucocytes as observed in leucemia and other blood disorders.<sup>18</sup>

The increased viscosity of this medium is advantageous in that the end point in filling the pipette may be reached accurately, there is little settling and clumping of white cells, and there is no tendency toward overflow in charging the chamber. The clear background of the counting chamber and the relatively slow rate of evaporation from it are also advantageous.

The differentiation of eosinophils in the same sample of blood employed in determining the total leucocyte count results in a minimum possible error in comparison with alternative methods.

Finally, the glycol stain technique also permits the counting chamber enumeration of polymorphonuclear and mononuclear leucocytes even in the presence of many young myeloid elements as observed in the course of acute allergic reactions following the ingestion of alleigene drugs<sup>10</sup>

The accuracy of the counting chamber enumeration of eosinophils on one side of the chamber (0.9 cmm) using the propylene glycol technique as compared with the standard method of enumerating eosinophils that is by determining their number as a result of performing 200 cell film differential counts and applying the percentage obtained to the total leucocyte count, was studied by Randolph and Stanton employing parallel observations from the same freely bleeding puncture wound. Such determinations were made in 100 normal persons and in 400 instances in allergic individuals who had varying degrees of eosinophilia. Finally, ten of the latter subjects were subjected to ten parallel eosinophil counts each by the two techniques.

Table I lists the maximum minimum mean and standard deviation determined as a result of the statistical analysis of these ten observations per subject by each of the two techniques.

TABLE I. EOSINOPHIL COUNTS AS DETERMINED IN ENUMERATING THE EOSINOPHILS IN ONE SIDE OF THE COUNTING CHAMBER (0.9 cmm) COMPARED WITH THE DIFFERENTIAL ENUMERATION OF EOSINOPHILS FROM PERFORMING 200 CELL DIFFERENTIAL COUNTS FROM THE STAINED FILM

SUBJECT	EOSINOPHILS IN 0.9 cmm OF CHAMBER WITH THE PROPYLENE GLYCOL TECHNIQUE				EOSINOPHILS FROM 200 CELL FILM DIFFERENTIAL COUNTS			
	MAX	MIN	MEAN	S. D.	MAX	MIN	MEAN	S. D.
Ch	110	44	68	27.0	113	0	61	46.0
H	710	422	562	83.4	722	420	553	100.0
G	799	555	673	92.0	1341	453	707	222.2
F	799	666	726	53.0	1278	402	076	229.4
M	977	888	932	39.8	1101	591	785	153.0
Q	1088	755	951	117.3	1155	552	849	204.0
Co	1379	755	1097	203.0	1411	981	1204	135.5
A.	2065	1121	1461	303.2	1937	1061	1448	249.2
E	1709	1332	1474	158.2	1725	1003	1397	234.5
B	1865	1221	1543	197.3	2219	1494	1950	216.0

From these studies it was determined that the enumeration of eosinophils on one side of the counting chamber (0.9 cmm) was a more accurate method of determining the number of these cells than the result of a differential count of 200 leucocytes from the stained film employing the cover slip technique and a standard method of sampling the stained area.

Henneman, Wexler and Westenhaver<sup>14</sup> recently compared the effectiveness of phloxine in equal parts propylene glycol in water (Solution II of this combined stain) with Thorin's modification of Drüger's hypotonic diluting fluid in enumerating eosinophils. In their experience with these two chamber techniques, the diluent composed of phloxine in propylene glycol not only provided more consistent results and no evidence of destruction of eosinophils but

also was preferred by them because of the ease in identification of cells, decreased evaporation of the diluent from the counting chamber, greater reproducibility of results, and because no refrigeration of the diluent was required. They confirmed our experience in using the eosin-acetone hypotonic diluent, commenting specifically on the rupturing of eosinophils in this medium and the large and rapid decrease in cell count with passage of time after dilution of the blood and with ageing of the oxalated blood.

Because of the more accurate enumeration of eosinophils permitted with the use of the glycol counting chamber stains, either the phloxine part of the diluent alone or the combined stain diluting fluid is preferred over alternative methods in following the levels of blood eosinophils in patients under treatment with adrenocorticotrophic hormone (ACTH).

#### SUMMARY

A white blood cell diluting fluid of phloxine and methylene blue dissolved in equal parts propylene glycol and water permits the counting chamber differentiation of eosinophils on the same blood specimen used in determining the total leucocyte count. In this medium the eosinophils stain bright red and are readily differentiated in the counting chamber from other polymorphonuclear and mononucleated leucocytes. This technique is the most accurate method of determining the number of eosinophils in the peripheral blood in that it permits the enumeration of intact stained cells in contrast to the previously described counting chamber techniques employing hypotonic diluents in which ruptured or "ghost" eosinophils are counted. It is a particularly valuable method to use in following the clinical course of patients under therapy with adrenocorticotrophic hormone (ACTH), those suffering from acute allergic reactions or infections in which one is primarily interested in the variations in serial determinations of the peripheral blood eosinophils.

This direct counting chamber technique makes it possible for the first time to express accurately the number of eosinophils existing in the peripheral blood, the expression of the number of eosinophils per cubic millimeter of blood is obviously more accurate and meaningful than to consider eosinophils in terms of the relative percentage of the total leucocyte count.

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# WARBURG MANOMETER CALIBRATOR\*

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## INTRODUCTION

IN CALCULATING the constant for a Warburg manometer it is necessary to know the volume of the Warburg vessel plus the volume of the manometer to the 150 mm mark. To determine the latter volume the manometer is usually filled with mercury to the 15 cm mark and the volume is determined from the weight and density of mercury. This method of calibration is very time consuming since it is somewhat difficult to introduce the requisite amount of mercury to just fill the manometer to the 15 cm mark. The manometer calibrator herein described greatly simplifies and shortens the calibration procedure. The principle employed is similar to that used in the construction of the Scholander microburette †

## CONSTRUCTION OF CALIBRATOR

The apparatus consists of a metric micrometer (Fig. 1 A) which is calibrated in hundredths of a millimeter. A stainless steel plunger (B) with a cross sectional area equal to 1 sq. cm (1.000) is pressed onto the micrometer spindle (C). A stainless steel collar (D) is pressed over the end of the micrometer. The upper end of this collar is threaded to accommodate the Lucite top (E). This plastic top is turned to a standard taper joint at H so that it fits the conventional Warburg manometer. A doughnut shaped "O" ring (F) fits over the plunger (B) and is compressed between the plastic (E) and the collar (D). This "O" ring seals the plunger within the plastic, yet it permits advancement of the plunger into the space (G).

## METHOD OF CALIBRATION

In use, the plastic cylinder is filled with water and the spindle is nearly withdrawn from the chamber (G). The cleaned manometer is inserted in the standard taper (H) of the plastic and fixed in place with rubber bands (Fig. 2). It is desirable to have just sufficient water in the calibrator chamber (G) so that only a small air bubble remains within the plastic cylinder when the manometer is in place. The manometer is held in the vertical position and the stopcock (M) of the manometer is opened and the other ends of the manometer are sealed by rubber tubes (N) and pinch clamps (O). The micrometer plunger is advanced and all the air is carefully expelled from the calibration chamber (G). The water meniscus is adjusted to coincide with the calibration mark of the manometer (J) (Fig. 2) (i.e. the mark used in calibrating the vessels) and the micrometer scale is read. The manometer is now turned to a horizontal position

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\*Manufactured by Micrometric Instrument Company, 7929 Kinsman Ave., Cleveland 4.

Ohio

†Scholander, P. F., Edwards, C. A., and Irvine, L. Improved Micrometer Burette J. Biol. Chem. 148: 195, 1943.



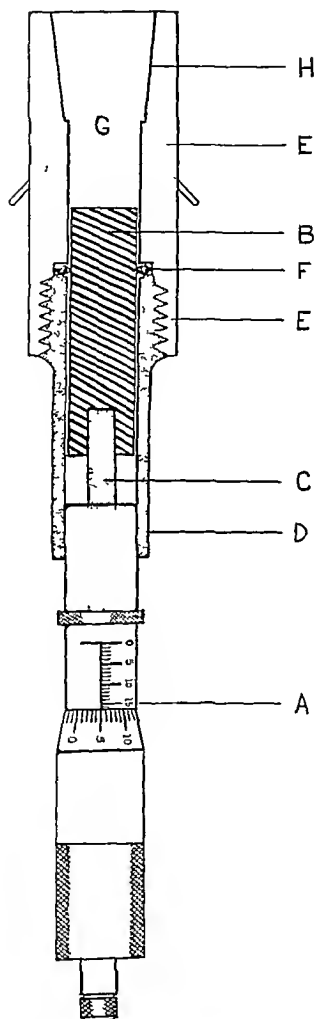


Fig. 1—Construction of Warburg manometer calibrator (for explanation see text)

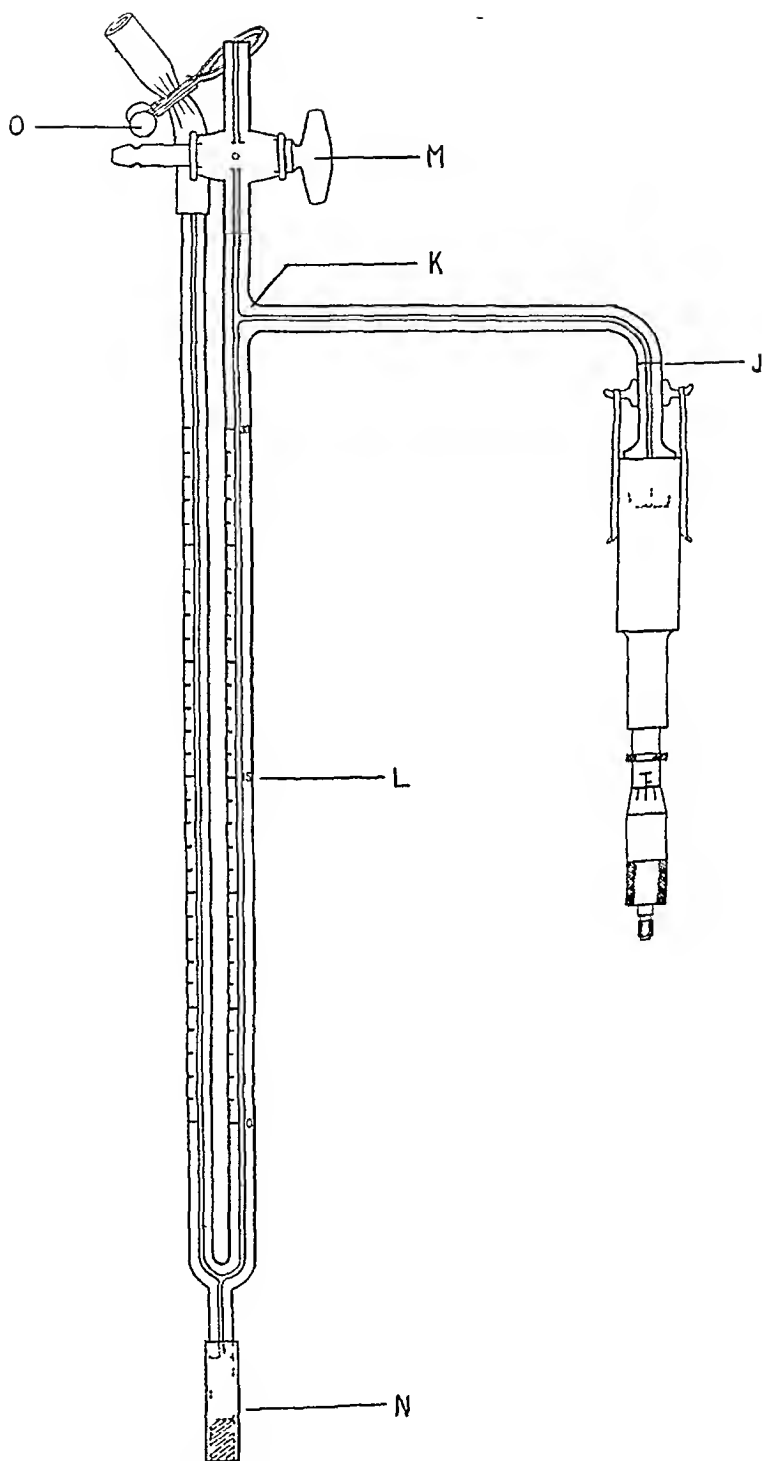


Fig 2—Calibrator inserted on Warburg manometer (for explanation see text)

and the plunger is advanced until the water meniscus reaches point *K* (Fig 2). Further advancement of the water meniscus fills the capillary between point *K* and the stopcock (*M*). When water reaches the bore of the stopcock (*M*), the stopcock is closed and the pinch clamp (*O*) is opened. The liquid is advanced to the 15 cm mark (*L*) of the manometer. The micrometer is again read and the volume determined by subtracting the initial from the final reading. The liquid is then removed from the manometer by reversing the foregoing procedure and the calibration may be repeated.

Results of several calibrations are shown in Table I. It will be noted that the volume of the manometer as determined in the first trial is slightly greater than that observed in subsequent trials. This difference is due to the residual water which is left in the manometer after the first calibration. It is further

TABLE I WARBURG MANOMETER CALIBRATION

REMARKS	TRIAL	VOLUME (C C)		
		3	8	34
Manometer dry	1	746	803	839
Manometer wet	2	720	787	820
Manometer wet	3	723	789	
Water left in manometers		025	014	019

noted that repeated runs on a wet manometer differ by less than 0.05 cubic centimeter. The accuracy of the volume measurement obtainable with this calibrator far exceeds the accuracy required for the calculation of the manometer constant. The accuracy of the calibrator itself may be checked by inserting a 2 cc pipette through a one holed rubber stopper and inserting the stopper into the standard taper (*II*) of the calibrator. Since the plunger has a cross sectional area of exactly 1 sq. cm., each millimeter advancement of the micrometer plunger corresponds to 0.100 cubic centimeter. Since it takes two revolutions of the micrometer to advance the spindle 1 mm. and since there are fifty small divisions per revolution of the micrometer, each small division of the scale of the micrometer corresponds to a volume of 0.001 cubic centimeter.

## SUMMARY

A simple rapid calibrator for the Warburg manometer is described.

## A NEWLY DEVELOPED ELECTROMAGNETIC FLOW METER

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THE general principle of an electromagnetic flow meter is not new. It was first developed by Kolin<sup>1</sup> in this country and independently by Wetterer<sup>2</sup> in Germany. Kolin<sup>3, 4</sup> developed both an A C model and a D C model, the latter was subsequently modified by Jochum.<sup>5</sup> These methods featured sleeves of various design which contained electrodes in positions so that when the sleeve surrounded a blood vessel, the electrodes were in contact with the outside surface of the blood vessel. While such devices possessed the advantage of not requiring incision into the blood vessel, they incurred the disadvantages of voltage drift and minor artifacts due to vessel drying, varying vessel diameter, and extraneous potential pickup. In order to avoid these disadvantages, a new method of potential pickup was developed in this laboratory which has proved exceedingly practical for the measurement of blood flow when used with an A C amplifier and suitable recorder. This newly developed electromagnetic blood flow meter has been found to be unusually accurate in its measurement of blood flow and to possess negligible voltage drift.

### PRINCIPLE

The basic principle upon which the operation of an electromagnetic flow meter depends is that when an electric conductor moves across the lines of force of a magnetic field, a potential difference is created in the conductor. If the field is uniform, the conductor moves in a plane at right angles to the magnetic field and the length of the conductor extends at right angles to both the field and the direction of motion, the resulting electromotive force will be directly proportional to field strength, speed of the conductor, and length of the conductor within the field. Therefore

$$E = B l v 10^{-8}$$

where  $E$  is the potential difference in volts,  $B$ , field strength in gauss,  $l$ , length of the conductor within the field in centimeters (across the electrodes), and  $v$ , speed of the conductor in centimeters per second. By use of the A C system, including an A C magnet, there is an additional constant to be accounted for because of the electrodes and their leads acting as a one turn transformer. Therefore, the total equation of an A C system must be altered to read as follows

$$E = B l v 10^{-8} + A$$

where " $A$ " voltage is the potential developed by the electrodes and leads, this voltage being proportional to  $B$  field strength. In order to cancel out a portion of " $A$ " voltage on the magnet a turn is made which is phased oppositely to that of the leads. The canceling potentiometer is turned in a manner to diminish " $A$ " voltage to a minimum. Once

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this point is established the voltage is of a constant and easily accountable magnitude. If  $B$  and  $l$  are kept constant  $K$  may be stated

$$K = Blv$$

and

$$F = K \lambda + A$$

As shown by the equations when the voltage  $\lambda$  is subtracted the electromotive force is a function of the single variable the velocity of flow which theoretically is linear. However, for practical considerations one is interested in volume flow in cubic centimeters per minute, rather than velocity flow in centimeters per second. The quantities are related as follows

$$Q = 60 \lambda \frac{2 \pi d}{4}$$

where  $Q$  is the volume flow in cubic centimeters per minute,  $\lambda$  the velocity flow in centimeters per second, and  $d$  the diameter of the tube or vessel. While this formula shows the relations of quantities it is not required for the use of the newly developed meter because due to the manner of its construction calibrations may be made of actual flow during an increment of time to read directly as cubic centimeters per minute blood flow.

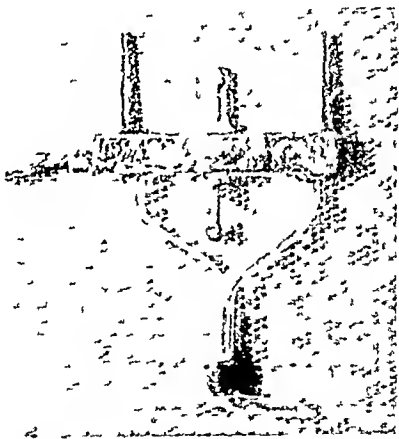


Fig 1—Cannula used with electromagnetic blood flow meter

#### MATERIALS AND METHODS

*Description of Apparatus*—The complete flow meter consists of a specially constructed cannula, an A C magnet, an A C amplifier, and an Esterline Angus & Mac recorder.

The construction of the cannula consists of a glass or plastic tube of previously determined diameter to equal the size of the lumen of the blood vessel to be cannulated. The cannula and pickup shown in Fig. 1 has a diameter of 3 millimeter. This particular cannula which is designed to fit the femoral artery of a dog is 4 cm. in height and 2.5 cm. in width. The bottom forms an anchorage to secure ligatures. Fig. 2 better reveals the detailed construction. In the top of the cannula are inserted two tungsten (platinum may be used) wire rods with rounded tips so that the tips just touch the periphery of the

lumen. Onto these tips just outside of the glass are soldered the two pickup leads which are then tightly twisted and passed through the center of the housing and into a shielded cable which runs to the amplifier connection. On the top of the housing are two pins which fit into the magnet case in order to assure identical positioning of the cannula during each measurement.

The housing is filled with Lucite for the purpose of stabilizing the position of the lead wires in the magnetic field. This is done by dissolving a portion of Lucite in  $\text{CHCl}_3$ , pouring the solution in the housing around the cannula and leads, and letting it congeal to form solid Lucite. This operation also makes the housing repellant to liquids. The

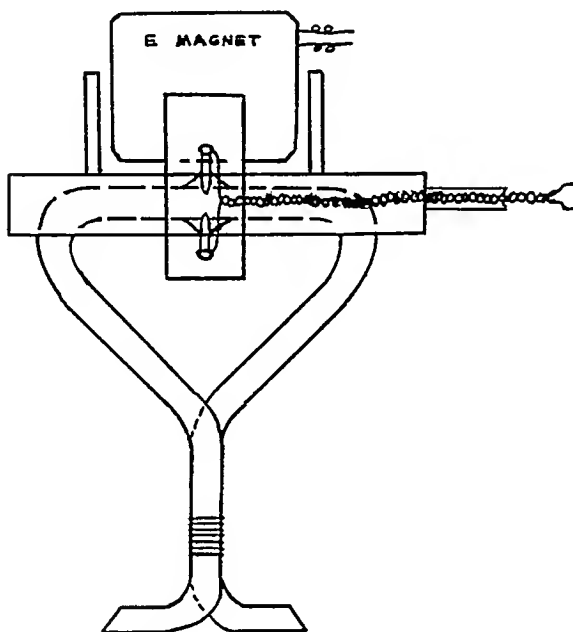


Fig. 2—Diagram of cannula and pickup leads showing the position of the magnet.

slender base of the cannula is wrapped tightly with a single layer of No. 8 linen thread and coated with the liquid plastic to add strength to the cannula. Care is taken in the construction of the cannula that there are no constrictions in the lumen and that there are no sharp bends. The short section across the top which holds the electrodes is straight.

The cannula and pickup incorporates the following features: (a) an electrical system well isolated from the surrounding tissues, (b) a lumen of uniform size to match the size of the lumen of the blood vessel measured, with no constrictions in its length, (c) a minimum of glass or plastic surface (less than four inches in length) with which the blood is in contact, (d) a reinforced slender base around which to secure ligatures surrounding the ends of the arteries, and (e) a specially designed bronze housing surrounding the electrodes through which the tightly twisted pickup leads are inserted to traverse the center of the magnetic field and be shielded from contact with the magnetic poles. It is the design of this housing which allows a minimal and invariable background pickup and thus prevents voltage drift on the recorder.

The A.C. magnet used with the meter is made from stacked laminations of  $\frac{1}{2}$  inch 29 gauge L4 audio A iron. The coil is made of 1,800 turns of No. 25 wire excited with a 110 V A.C. 60 cps source. This gives a field of about 1,000 gauss across a  $\frac{1}{2}$  cubic inch air gap between pole tips. A 60 cps power source limits the frequency response of the whole system to fluctuations of less than 60 cps, but it is possible to modify this limitation by changing the carrier frequency to 400 cps if desired. Should the meter be used

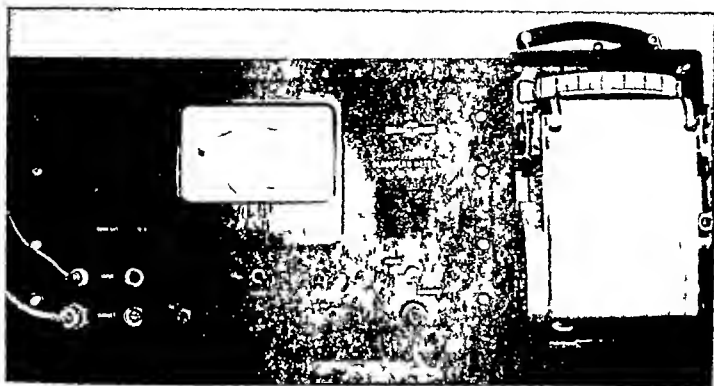


Fig 3.—Recorder and amplifier showing the connections as labeled on the panel

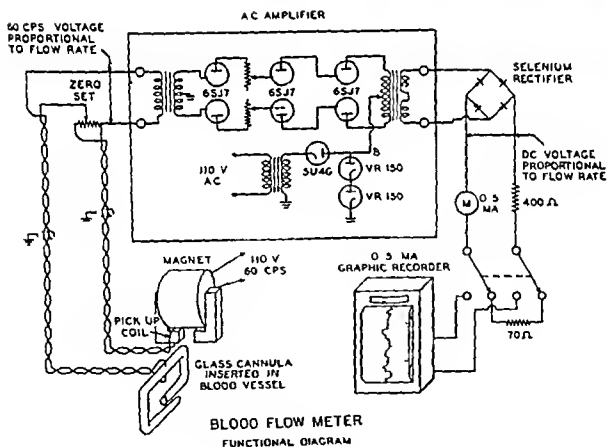


Fig 4.—Simplified diagram showing the electromagnetic pickup amplifier and recorder

with a high frequency response recorder, this modification is recommended. For convenience in adjusting the position of the magnet, a screw type lift with a crank attached is used to slowly lower the magnet into position over the cannula. The magnet is held by a ball in socket swivel which will allow it to be adjusted at a convenient angle.

The AC amplifier and recorder used are shown in Fig 3. The amplifier is adapted from the original AC amplifier of Kohn with minor modifications. Three stages of 6SJ7 tubes are used for the principal circuit with two VR 150 tubes being used on the B+ circuit

This amplifier has proved remarkably stable and features two input channels for pickup, each connected to a potentiometer to be utilized in canceling out stray background. A selenium rectifier is used in the output circuit. Any stable amplifier with sufficiently high gain would be adaptable for the electromagnetic flow meter, so long as it matches the characteristics of the pickup and the magnet used. This particular amplifier is most adaptable to a 60 cps magnet. The amplifier used here has two gain settings, the two amplifications giving a total range of blood flow measurement from zero to 300 cc per minute with different cannulae. The range of measurement may be increased by constructing the

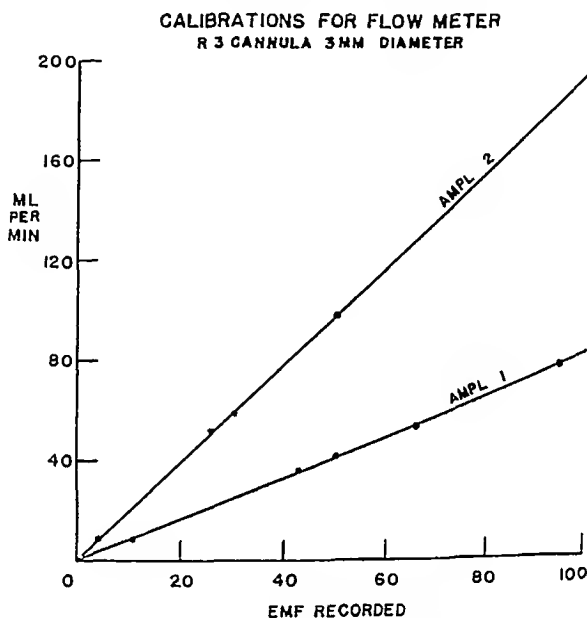


Fig 5—Sample calibration curve showing the use of two amplifications with one cannula

electrodes of the cannula so that the distance between the tips is diminished. Conversely, greater sensitivity is achieved by constructing the electrodes of the cannula so that the distance between the tips is greater. In all cases the lumen of the cannula should be as nearly as possible identical with the size of the blood vessels.

A 5 M Esterline Angus recorder is used on the electromagnetic flow meter as a compromise choice of various possibilities. This instrument requires 5 M $\Omega$  through an internal resistance of 70 ohms for full scale deflection. This is a heavily damped instrument and will not record events accurately under 200 milliseconds but this feature is of value in the recording of mean blood flow where a concomitant record only of heart rate and respiration is desired. When events of shorter duration are of interest, a recorder of higher frequency response should be employed. Fig 4 reveals a functional diagram of the entire apparatus as it is operated showing the position of the magnet, the general structure of the amplifier, and the recorder connections.

*Calibration of the Instrument*—Although a force pump perfusion system has been used to calibrate the flow meter, it is felt that the preferred method is to measure the flow of blood out of a blood vessel directly into a graduate over a convenient period of time. This method is to be desired for its simplicity and the elimination of extraneous intervening variables. In operation, the distal end of the cannula is attached to a plastic tubing of the same size which runs to a graduate. A previously applied clamp on the proximal artery is released for one minute so that a one minute flow of blood is spilled into the graduate. A point is made on the midline of the excursions on the record which corresponds to the milliliters of blood



measured. This is one point on the calibration curve. By constricting the end of the plastic tubing, lower rates of flow may be measured until a number of desired points on the flow curve may be calibrated. A curve of best fit may be drawn transecting the points and may be extrapolated to the base line. This point on the base line or true base will not fall on zero, but upon an electromotive force reading, which represents the "A" voltage previously described. These readings may be made identical, by means of the potentiometer adjustment. Fig. 5 shows typical calibration curves by use of two amplifications. In this calibration graph the "A" voltage has been subtracted and it may be seen that the curves are for all practical purposes linear.

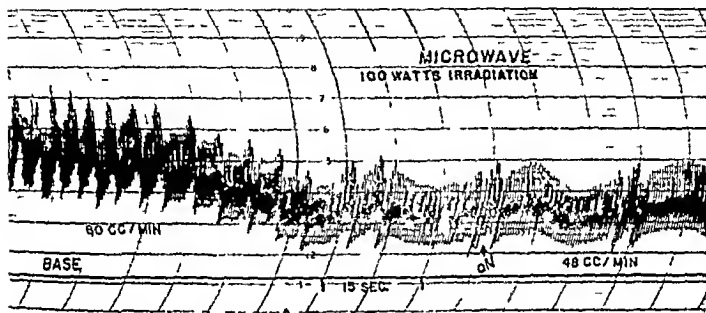


Fig. 6—Sample record made with the new electromagnetic flow meter. The record reads from right to left.

Once it is calibrated, a cannula does not require recalibration unless it develops a fracture or similar defect. However, because of such possibilities, frequent recalibration may be advisable. The typical observation with a fractured cannula is an enormous base voltage, which ordinarily can be detected readily.

#### RESULTS

Fig. 6 shows a typical blood flow record which reads from right to left. This sample record shows the results of 12 cm. microwave irradiations using 100 watts output upon the limb of a dog. The wave director was placed close to the surface of the limb. In the figure, the heavily inscribed base line represents the background pickup, or "A" voltage, so that voltage induced by blood flow reads from this point to the midline of the excursions to represent mean flow. This point at the midline was arbitrarily selected when calibrating the cannula. When this distance is read on the ordinate and multiplied by the  $k$  value of the cannula in this case ( $20 \times 10$ ), a figure is derived which represents blood flow in cubic centimeters per minute. For example, the control flow may be calculated as follows:

$$0.24 \times 20 \times 10 = 48 \text{ cc/min}$$

The calibration should be experimentally established as suggested, and the "A" voltage reading may be conveniently adjusted. Since the "A" voltage will be  $90^\circ$  out of phase with the flow voltage, they cannot be added algebraically.

Fig 6 is a reproduction of blood flow in the femoral artery. The rapid oscillations are the result of the cardiac rhythm, while the slower oscillations are due to respirations. These events are reflected in the flow record with sufficient clarity that the record may be used to count the respirations and heart rate. It may be observed that while the rate of blood flow and respiration have been increased by the irradiations, there is not noticeable increase in heart rate. This record was made at one of the intermediate speeds. A faster speed makes the excursions much easier to analyze, while a slower speed achieves a greater economy of record.

#### DISCUSSION

While it is essentially a pulsatile flow meter, this device as described here is best adapted for the measurement of mean blood flow. As previously suggested, it is possible to better adapt this meter for the measurement of pulsatile blood flow where a finer analysis of events of a short duration is desired.

The meter as described has proved exceedingly capable for the measurement of mean blood flow in both veins and arteries. It has been found to possess less than 5 per cent error in measurement under physiologic conditions and has been operated for four- to six-hour periods with negligible voltage drift. A criticism of this type of flow meter is that general anesthesia and an anticoagulant must be used because the blood vessel is cannulated. On the other hand it is simple to operate, is substantially free from subjective factors, and possesses a relatively linear calibration curve. By use of different cannulae, the range of flow measurement is from 0 to 300 cc per minute. This range may be expanded with simple modifications. The meter may be easily cleaned following each use by means of a detergent and distilled water. This cleaning, carefully done, is important for proper measurements.<sup>6</sup> In brief, while the development of this meter entailed certain compromises it is felt that it has proved to be a practical, accurate, and easily operated device for the measurement of blood flow under many conditions encountered in laboratory measurement.

#### SUMMARY

A new electromagnetic blood flow meter has been developed which produces a continuous permanent record of mean blood flow. This device is adaptable to measure pulsatile blood flow in the respect that it readily records the effect of the heart rhythm and respiration upon blood flow. Modifications are suggested for recording events of shorter duration. The use of this meter entails the disadvantages inherent with cannulation, but such disadvantages must be weighed against the advantages of accuracy, stability, and ease of operation. The stability and accuracy of this meter is in a large measure dependent upon careful construction of the cannula and pickup leads.

The authors gratefully acknowledge the cooperation and assistance of Dr J W Clark in the development of this work.

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# EVALUATION OF A NEW CAPILLARY RESISTOMETER THE PETECHIOMETER

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## INTRODUCTION

CAPILLARY resistance determinations were made on one hundred patients with two different types of suction apparatus and the readings compared. A modified Dalldorf resistometer was used on one forearm as previously described,<sup>1</sup> and a new resistometer, the Petechiometer,\* was applied to the other forearm.

## MATERIALS AND METHODS

Capillary resistance is the minimal amount of suction applied to the skin for one minute capable of producing one petechia or more. It is measured in centimeters of mercury negative pressure or suction. With the modified Dalldorf resistometer, readings were made at intervals of 5 cm, the amount of suction being read directly from a vacuum gauge. The Petechiometer, on the other hand, has no vacuum gauge but is adjusted to deliver 10, 20, and 30 cm of mercury suction. The bell of the old resistometer is 1 cm in diameter while that of the Petechiometer is 2 centimeters.

In all of the one hundred patients two readings were noted, the old instrument being used on one forearm and the Petechiometer on the other, Fig 1. Because in some studies<sup>2, 3</sup> readings were normally slightly lower on the right arm, it was decided to reverse machines in the second group of fifty patients. The 200 capillary resistance readings may be compared in Table I.

*Estimation of Capillary Resistance With the Petechiometer*—The plunger is grooved at three points, each yielding a different suction. The adjustable stop ring is inserted in the innermost groove. One then places water just below the antecubital space before applying the bell to the wet skin. The air is expelled by pressure of the right thumb on the end of the plunger and the bell placed on the skin. The bell is held in contact with the skin lightly, but firmly, with pressure from the index finger of the left hand upon the top of the bell. As the thumb pressure on the plunger is released quickly a spring action applies suction to the skin area. Suction is applied for one minute and then released by pushing inward on the plunger. After removing the Petechiometer, thirty seconds are allowed before looking for petechiae. If no petechiae are found, the test is repeated using each of the stop-ring settings, i.e., the innermost yielding 10 cm mercury suction, the middle setting 20 cm mercury suction, and the outermost slot setting 30 cm mercury suction. The determinations are not made closer together than the diameter of the suction bell.

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\*The Petechiometer is the registered trade mark owned by the Rexall Drug Company. The Petechiometer consists of a small suction pump with a plunger and a clear plastic Bell or suction cup 2 cm in internal diameter with a magnifying glass blown into the upper surface of the cup to facilitate in situ reading of the petechiae developed.

The number of petechiae produced by the Petechiometer was noted. In the final comparison of results this procedure proved valuable, for it permitted interpolation to intervals of 5 cm when excessive numbers of petechiae were produced at the 10, 20, or 30 cm readings. For example Patient 29 showed a capillary resistance of 10 cm on the Petechiometer but had forty petechiae

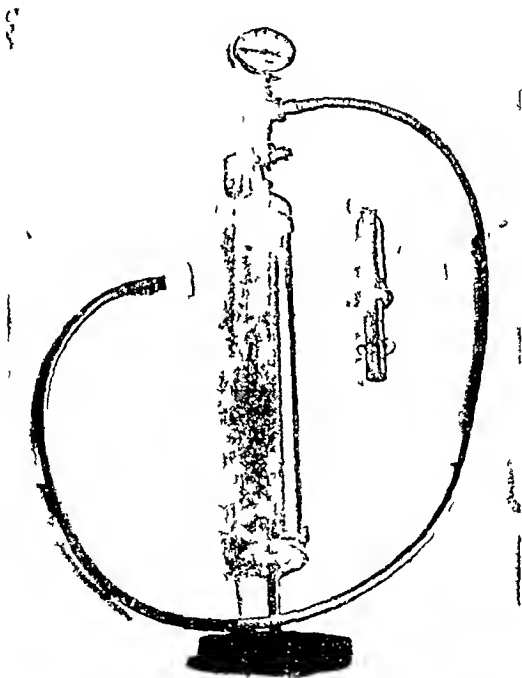


Fig 1—Comparison between old and new resistometers

Obviously, because of the many petechiae produced the true capillary resistance was actually less than 10 centimeters. Using the old resistometer the capillary resistance in this patient was 5 centimeters. When comparisons were made with the two machines it was found that when six or more petechiae were produced by the Petechiometer, one could in most instances subtract 5 cm from the capillary resistance shown by this new instrument and approximate the reading given by the old machine.

TABLE I CAPILLARY RESISTANCE READINGS (CENTIMETERS OF MERCURY SUCTION) IN 100 PATIENTS WITH NEW AND OLD RESISTOMETERS, USING RIGHT AND LEFT FOREARMS

	NEW RIGHT			OLD LEFT		NEW RIGHT			OLD LEFT		NEW LEFT			OLD RIGHT		NEW LEFT			OLD RIGHT
	*	P	†			*	P	†			*	P	†			*	P	†	
1			20	15	26			10	10	51			30	15	76			30	30
2	10	8	5	5	27			20	10	52			10	10	77	20	8	15	15
3			10	5	28	10	12	5	5	53			20	20	78	10	8	5	5
4			20	15	29	10	40	5	5	54	10	20	5	10	79	10	7	5	5
5			10	10	30			20	25	55			10	10	80	10	8	5	15
6			20	15	31			10	10	56			10	15	81			10	10
7			20	15	32			30	15	57			10	15	82			10	15
8	20	8	15	20	33			10	10	58			20	20	83	10	7	5	10
9			20	15	34			20	25	59			10	15	84			20	15
10			20	25	35			20	15	60			10	5	85			10	10
11			10	15	36			20	15	61			10	5	86			10	10
12			20	20	37			20	15	62			10	15	87			20	10
13			10	10	38	10	7	5	10	63			20	15	88	10	6	5	5
14			20	25	39	20	6	15	20	64			20	15	89	10	8	5	10
15			20	20	40			10	15	65			10	10	90			10	10
16			20	15	41	30	14	25	25	66			10	10	91			15	20
17			20	15	42			20	25	67	20	8	15	15	92			10	10
18	20	8	15	30	43	20	8	15	15	68	10	6	5	5	93			10	10
19			30	25	44			10	10	69			20	25	94			10	10
20	20	12	15	25	45	20	8	15	15	70	20	8	15	15	95			10	15
21			10	10	46			10	20	71	20	8	15	15	96			30	20
22			30	35	47			20	30	72			20	25	97			10	15
23			20	25	48			10	10	73	20	8	15	15	98	20	16	15	5
24			10	15	49	10	6	5	5	74			20	20	99	10	17	5	5
25			10	10	50			10	5	75	20	15	15	15	100			20	15

\*Capillary resistance before 5 cm correction for excessive number of petechiae (six or more)

P Number of petechiae

†Corrected capillary resistance

## RESULTS

By subtracting 5 cm when six or more petechiae were noted at the capillary resistance determination with the Petechiometer, the average reading with the old instrument was 14.75 cm and with the Petechiometer 14.25 centimeters. This similarity is not so constant in individual readings. Only 43 per cent showed no difference between old and new resistometers and 90 per cent did not vary more than 5 centimeters. Of the ten patients who differed by more than 5 cm between arms, seven patients showed a 10 cm difference and three patients a 15 cm difference. One may expect, however, to find similar variations between arms even when using the same resistometer.<sup>2, 3</sup>

## DISCUSSION

A simple capillary resistometer to determine bleeding tendencies should be welcomed by physicians. It has been noted that the lower the capillary resistance, the greater is the bleeding time.<sup>4</sup> A capillary resistance of 5 cm means marked fragility. Capillary fragility accounts for bleeding in such conditions as purpura and rheumatic fever and in hypertensive patients.<sup>3, 5, 6</sup>

Without a suction apparatus for determining capillary resistance, the clinician may resort to positive pressure methods, as by use of the sphygmomanometer.<sup>7, 8</sup> The disadvantages of such methods are listed by Tey<sup>9</sup> as follows: (1) determination is time consuming, (2) test cannot be repeated for days,

(3) there is no gradation in the same subject (4) results cannot be read with accuracy, (5) positive pressure methods are unsuited for pharmacologic investigation, where several determinations must be made within a few minutes since only two body surfaces (arms) are available

On the other hand use of the Petechiometer is simple and painless. Determination of capillary resistance requires less than five minutes. The instrument is small and can be carried in one's bag.

#### CONCLUSIONS

Capillary resistance determinations were made on one hundred patients using a modified Dalldorf resistometer with vacuum gauge on one forearm and a new instrument, the Petechiometer on the other.

Although readings on both forearms differed in individual subjects, these differences were usually slight. Only 10 per cent of patients showed readings which differed more than 5 cm. between arms. Minor differences are normally noted between arms. These differences tended to balance in the final compilation for the average capillary resistance with the old machine was 14.75 cm. and with the Petechiometer 14.25 centimeters. The Petechiometer thus appears to be accurate.

The Petechiometer has the following advantages over the older resistometer: it is on the market, it is simple to use, it is compact and small.

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# A SIMPLIFIED VENOUS OCCLUSION METHOD OF DIGIT BLOOD FLOW ESTIMATION USING THE BURCH-WINSOR PLETHYSMOGRAPH

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**D**IGIT blood flow studies based on the venous occlusion principle have been greatly simplified by the development of a clinical type of transmission plethysmograph by Burch.<sup>1</sup> At the present time venous occlusion blood flow determinations are being carried out as a part of the regular digit plethysmographic study on both fingers and toes in our laboratory. The basic details of this type of study have been recorded by Wilkins,<sup>2</sup> Goetz,<sup>3</sup> Abiamson,<sup>4</sup> and others.<sup>5</sup> Our purpose in reporting this method is to emphasize the ease with which venous congestion methods may be adapted to the Burch-Winsor plethysmograph. Measurements with this type of apparatus depend upon volume changes in the part under study, the variables being amount and rate of volume change. For the most part an increase in the volume of a trapped part is dependent upon either increased arterial inflow or decreased venous outflow or both. If one occludes the venous outflow without disturbing the arterial inflow the volume change resulting represents an indirect estimate of the arterial inflow. The inherent limitations of this type of determination have been adequately discussed by Abiamson and by others.<sup>2, 3, 4, 5</sup> We are aware of these limitations, but with increasing experience we are coming to feel that digit flow determinations are worth-while adjuncts to digit plethysmography, particularly in attempting to decide about the presence or absence of vasomotor activity in the digital blood supply.

The problem of applying an occluding pressure to the base of the digit might be mentioned first. Our first attempt was a brass-backed latex ring. This was a satisfactory method for fingers but in the case of toes a ring large enough to pass over the end of the toe was frequently too large to satisfactorily compress the toe at its base. The brass-backed latex cuffs were discarded in favor of Abiamson's adhesive tape-latex tube method. In making the latex tubing for the occluding cuffs a glass rod 6 mm. in diameter which has been dusted with talcum powder is dipped into a container of prevulcanized liquid latex two or three times. After several hours of drying, the surface of the rubber on the glass rod is dusted with talcum powder and the rubber is rolled off the glass. The tubing thus obtained is thin enough to avoid bulk and strong enough to withstand the occluding pressures. The latex tubing is attached to a small catheter or plasma tubing with a fine silk binding reinforced with a turn of adhesive tape. The latex tubing is then backed with ordinary adhesive tape.

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The end is turned under and covered with a tab of adhesive. The completed cuff is 8 to 10 cm in length and approximately 1 cm wide\*. These cuffs, if well made and free of bubbles, will survive a week or more of active use. In applying the cuff to the digit the cuff is wrapped around the base of the digit and a short piece of adhesive is used to secure the cuff to itself to prevent slipping (Fig 1 A and B).

The method of cuff inflation used is a simple pressure bottle arrangement similar to that of Goetz<sup>3</sup>. The essentials of the pressure system are a 1 or 2 liter widemouthed bottle, a tightly fitting rubber stopper, a large three way glass stopcock, some type of manometer—either aneroid or mercury, a blood pressure type of compression bulb and suitable tubing and glass or metal connections. The illustrations (Fig 2 A and B) are self explanatory and give a satisfactory scheme of connections for use.

In use the occluding pressures should be well below diastolic arterial pressure and we have found 60 to 70 mm (Hg) to be satisfactory for most digits. When air is allowed to flow into a single cuff the others having been excluded by clamps the bottle pressure falls 1 to 2 mm of mercury with each inflation. This is corrected prior to each occlusion by means of the compression bulb.

To summarize the steps in the digit flow determination

- 1 The digit part is measured in the displacement tube apparatus<sup>1</sup> to a volume of 5 cubic centimeters. This volume is indicated on the digit by a weak gentian stain which is employed as the measuring fluid.

- 2 The extremity plastic cup is sealed in place at the proximal edge of the gentian stain with the sealing compound Kilk Kord previously reported.

- 3 After the cup seal has been tested the occluding cuffs are applied to the base of the digit in such a way that there is no contact with the cup or the sealing compound—and no contact between the tubing from the cup and cuff. Trial cuff inflations are then carried out to make certain the cuffs are in the best alignment to prevent unnecessary motion of the digit when the cuff pressure is introduced.

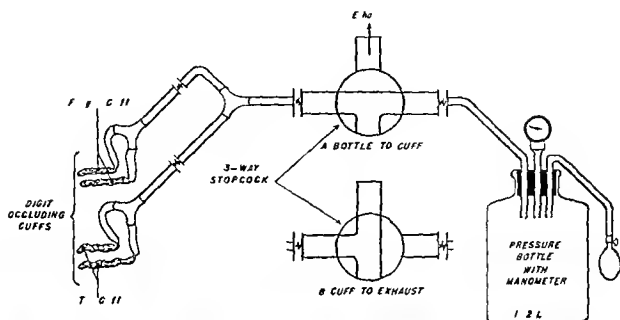
- 4 String sensitivity is adjusted to move 10 mm per 10 mm<sup>3</sup> volume change with the calibration lever. The routine plethysmographic tracings are made at slow speed standardizing each run twice. Following the second calibration, the base line is shifted to the left side of the aperture and the camera speed shifted to 'fast' just prior to the application of cuff pressure. This will require a certain amount of practice by operator and assistant. When the maximal base line shift (i.e. volume change) has occurred the camera is shifted to slow speed and cuff pressure is released by turning the stopcock to the 'exhaust' position. The base line is adjusted to the left side of the aperture if necessary following which the venous occlusion test is repeated. The camera is then stopped and the next digit is prepared for recording. With practice the volume pulses and two venous occlusion runs can be recorded on about 12 inches of paper for each digit.

\*The latex which we have found satisfactory for this use is a formula designated No. G.L. 1 furnished by the General Latex and Chemical Corp. 666 Main Street, Cambridge, Mass.

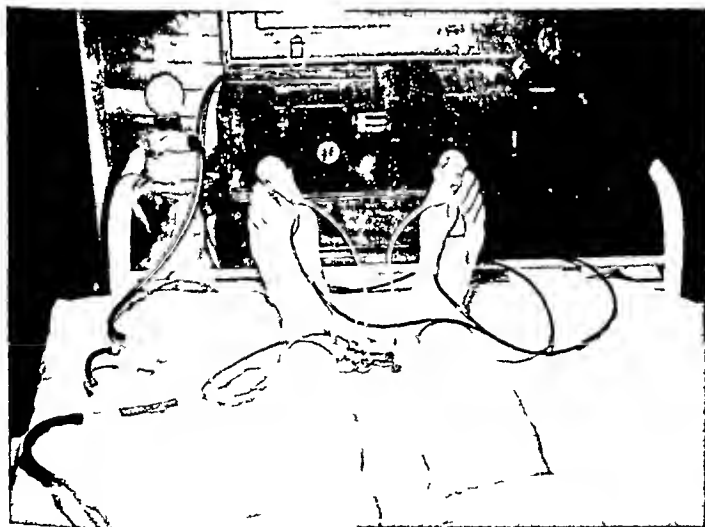
*A**B*

FIG. 1—See text for explanation

DIAGRAMMATIC SYSTEM  
OF CUFF-PRESSURE BOTTLE CONNECTIONS



1



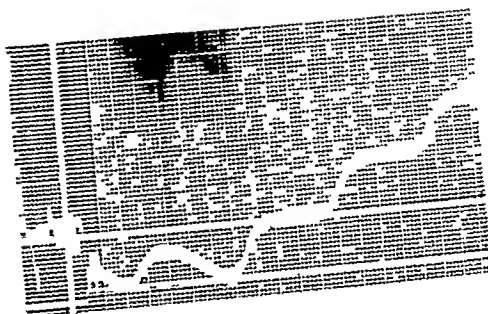
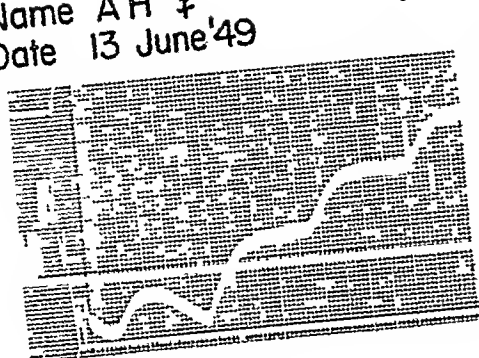
B

Fig

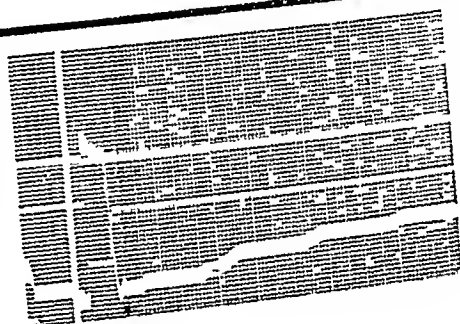
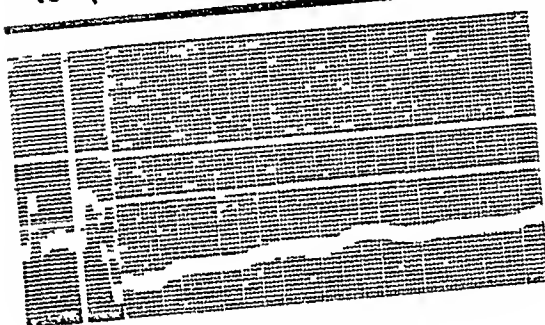
# Venous Occlusion Test

Digit Left 1st Toe

Name AH ♀  
Date 13 June '49



Warm Room  
Temp 83°F



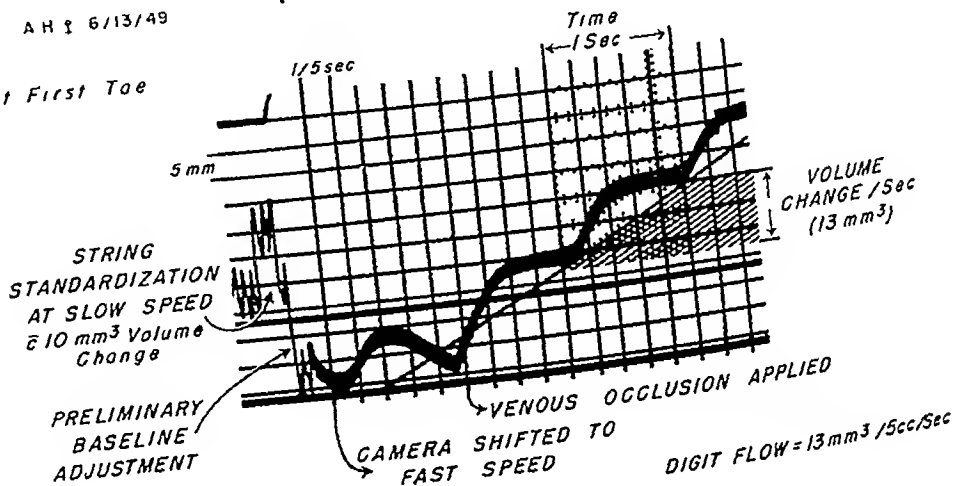
Cold Room  
Temp 68°F

A

## ESTIMATION OF DIGIT FLOW (mm<sup>3</sup>)/5 cc part/sec

AH ♀ 6/13/49

Left First Toe



B

Fig 3

5 After the tracing has been developed the digit flow can be readily determined by measuring the amount of volume change per unit of time (Fig 3, B). We have arbitrarily used millimeters' per second per 5 cc of part. In drawing the tangent slope line it is desirable to avoid using the first pulse excursion if possible since positive or negative artifacts are common. In rapid flows this may not be possible because of the speed with which the string shadow crosses the entire width of the paper in which case the slope line should be placed along the most representative portion of the curve. In connection with measuring the various string deflections on the tracing a correction formula has been used to compensate for "high or low calibration errors. The correction ratio which we employ uses the following factors:

$$\frac{\text{Measured volume deflection}}{\text{Measured calibration deflection}} = \frac{\text{True volume deflection (X)}}{10}$$

Thus if the measured volume deflection were 14 mm and the standardization deflection were found to be 8 mm instead of 10 mm the measured deflection would be low and by the correction formula the true volume deflection would be

$$\begin{aligned} 14 \quad X &= 9 \quad 10 \\ 8X &= 140 \\ X &= 1.50 \text{ mm/5 cc of part (corrected deflection)} \end{aligned}$$

Although this formula does not take into consideration all the variable factors which might cause minor errors, such as variation in volume of the trapped part, it is probably worth while in measuring volume pulses and blood flow where the standardization error is greater than 1 millimeter.

The illustrations demonstrate the essential details of this method of blood flow estimation in digits. The plethysmograms presented are tracings from normal individuals who have no evidence of peripheral vascular disease. The "warm room" flows have been recorded after one hour in an ambient temperature of 83° to 84° F and the "cold room" flows after one hour in an ambient temperature of 68° to 69° F. (Fig 3 A and B).

The application of this type of digit blood flow determination to normal subjects and before and after limb sympathectomy will be presented in subsequent communications.

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# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

*Twenty-Second Annual Meeting*

*Chicago, Ill , Nov 4 and 5, 1949*

## ABSTRACTS—Concluded

### 61 EXPERIMENTAL PRODUCTION OF MEGALOBlastic ANEMIA, AN INTERRELATIONSHIP BETWEEN ASCORBIC ACID AND PTEROYLGLUTAMIC ACID

CHARLES D MAY, M D , E N NELSON, M D (BY INVITATION), AND  
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This report describes a means of producing megaloblastic and macrocytic anemia in monkeys using deficient diets alone

The diets employed were basically dried cow's milk reconstituted with water and adjusted with lactose and vegetable oils and supplemented with crystalline vitamins to provide liquid diets of the following approximate composition

	PER 100 ML
Protein	16 Gm
Carbohydrate	72 Gm
Fat	33 Gm
Calories	67
Vitamin A	800 I U
Vitamin D	50 I U
Thiamin	07 mg
Riboflavin	1 mg
Nicotinic acid	5 mg

Any other vitamins present were those contained in the milk Each experimental animal was given 137 mg Fe per kilogram in the form of non ascorbate intravenously at the start of the experiment followed by several weeks of oral therapy with 4 cc of ferrous gluconate daily

In five control periods monkeys fed this type of diet ad libitum and 50 mg ascorbic acid daily for five or more months remained in good health and did not develop anemia or changes in the bone marrow

When the experimental diet was fed to eleven monkeys without the addition of ascorbic acid, a typical sequence of events occurred For three to four months they remained in apparent good health At about this time the first symptoms of scurvy appeared They remained essentially in this state for the next three to four weeks, exhibiting only the symptoms of scurvy, without developing anemia or definitive changes in the marrow Then a precipitous deterioration began Pronounced anorexia and listlessness ushered in this phase Diarrhea commenced invariably, the stools being loose to mushy, bulky and foul but without pus or blood With one exception a histamine re fractory gastric achlorhydria developed The fur lost its smoothness and luster and was shed in large amounts The periodontal gingivae became ulcerated and necrotic No changes were seen in the tongue No infections were observed in the animals at any time Anemia and neutropenia developed rapidly without evidence of additional hemorrhage The anemia was usually of a normocytic or macrocytic type

The most critical criteria were applied in designating the types of cells seen in the marrow Three monkeys developed megaloblastic marrows very similar to, if not identical with, those seen in megaloblastic anemia in human

benigs The marrows in the other eight animals became megaloblastic, a stage through which the megaloblastic marrows passed The alterations in the granulocytes in each instance were striking, showing hypersegmentation and large curly forms with premature lobulation such as are seen in the marrow in pernicious anemia It was the impression that all the marrows might have progressed to a frankly megaloblastic picture if the pernicious condition of the animals had not made it seem necessary to treat them in order to test the effects of various agents

A systematic trial of vitamin B<sub>12</sub>, PGA and ascorbic acid revealed the following (1) Ascorbic acid given relatively early in the disease gradually restored the animal to health and the marrow and blood became normal (2) If ascorbic acid was not given until an advanced stage of the disease it did not restore the marrow to normal or prevent the death of the animal (3) Vitamin B<sub>12</sub> alone given intramuscularly had no effect on the marrow within forty eight hours (4) PGA given orally or intramuscularly as the free acid or the triglutamate but without ascorbic acid promptly stimulated normal hematopoiesis in the marrow and relieved all the symptoms not attributable to scurvy When ascorbic acid was added normal health was restored even though the experimental diets were continued

Increasing the protein in the diet to 3.3 Gm per 100 cc by adding calcium caseinate did not prevent the development of the characteristic anemia

Thus it would appear that megaloblastic anemia developed as a result of PGA deficiency somehow induced by a chronic deficiency of ascorbic acid

## 62 CERTAIN EFFECTS OF CHEMOTHERAPY ON THE FECAL AEROBIC AND ANAEROBIC BACTERIA OF PATIENTS WITH CHRONIC ULCERATIVE COLITIS

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AND JOSEPH B KIRSNER MD CHICAGO ILL

The role of the fecal bacteria in the pathogenesis and course of chronic nonspecific ulcerative colitis is not clear A method of simplifying the bacterial flora of the feces and reducing the number of bacteria in the bowel would provide useful information concerning this problem Accordingly, qualitative and quantitative studies of the aerobic and anaerobic bacteria were made before and after treatment with absorbable and nonabsorbable sulfonamides penicillin administered orally and parenterally and the oral administration of streptomycin aureomycin and chloromycetin Control studies consisted in observations intended to determine the error of the method and to ascertain the spontaneous day to day variation The drugs were administered for relatively long periods of time bacteriologic studies were carried out at frequent intervals in order to determine the initial and late effects of chemotherapy All of the drugs under certain conditions, are capable of altering the aerobic flora some also may modify the anaerobic flora of the feces the degree and duration of effect varying considerably For instance, sulfonamides alter the aerobic flora, when initially used for periods as long as six weeks Streptomycin, on the other hand exerts a marked quantitative effect which is maintained however only for several days Chloromycetin and aureomycin exert an intermediate effect the *B coli* disappear and the total bacterial count initially falls and then rises with the reappearance of *B coli* as a predominant organism In addition after the initial depression of bacterial counts had terminated the counts tended to rise above the control levels and to remain high decreasing to control levels only after the drugs were discontinued

All of the patients improved when a basic regimen was instituted which provided a diet which contained calories equivalent to twice the calculated basal requirement,  $2\frac{1}{2}$  Gm of protein per kilogram of body weight, 30 per cent of the calories as fat and the remainder as carbohydrate. The patients' clinical improvement was accelerated by the addition, to this regimen, of daily intravenous infusions of a mixed amino acid solution which was sodium free.

Restriction of the sodium intake below 10 Gm per day was a valuable adjuvant in combating fluid retention. The patients with edema and ascites showed an increased urinary output and the edema and accumulation of ascites diminished when salt intake was restricted.

No clinical benefits were observed in the patients when the basic regimen was supplemented with cystine, methionine, choline, B complex vitamins, or parenteral liver.

The infusion of large amounts of concentrated human serum albumin (salt pool) was impractical as a method of protein supplementation, even though a transient rise in serum albumin concentration and a marked increase in positive nitrogen balance attended its administration.

On the regimen described (diet, salt restriction, and supplementation with amino acid infusions) all of the patients showed increased weight gains without fluid retention, those with edema and ascites gradually showed reduction and disappearance of these manifestations of liver disease. All patients showed a striking capacity for the retention of tremendous amounts of nitrogen, probably due to replacement of fluid in the tissues by newly synthesized muscle protein.

One patient with advanced cirrhosis and recurrent ascites was observed over a period of two years while subsisting on seven different regimens. At the peak of the patient's illness, paracenteses were necessary at ten to twenty day intervals and 16 to 19 liters of fluid were removed at a time. Albumin therapy produced no alteration in the rate of accumulation. A low salt regimen reduced the frequency of paracenteses to about thirty-day intervals. Finally, with the previously described regimen, the patient became ascites free and has remained so for 7 months. He has maintained his body weight gains and has returned to a normal active existence.

Of seven liver function tests, serum cholinesterase activity was the most reliable test of liver function in that the cholinesterase levels most accurately correlated with the patient's clinical condition.

## 67 QUANTITATIVE STUDIES OF VIBRATORY PERCEPTION IN DIABETIC AND NONDIABETIC SUBJECTS

I ARTHUR MIRSKY, M.D., PERRY FUTTERMAN, M.D. (By Invitation), and  
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Quantitative measurements of the threshold of vibratory perception were made in 102 diabetic patients and in 136 nondiabetic, nonobese, nonhypertensive subjects. Both groups of patients were chosen at random from an ambulatory population and were from 5 to 79 years of age.

No correlation was found to exist between the threshold of vibratory perception and the side of the body tested or the sex of the subject. A significant increase in the threshold was found in the diabetic group of subjects. This increase was not related to either the known duration of diabetes mellitus or the severity of the metabolic derangement as estimated by the patients' insulin requirements. Diabetic subjects with symptoms or signs of neuropathy tended to have a greater incidence and greater degree of vibratory



impairment than those without such signs or symptoms. Similar measurements in nondiabetic groups of obese and hypertensive patients revealed no differences in their thresholds from those of the control group.

Both the diabetic and nondiabetic groups of subjects showed an increasing threshold of vibratory perception with increasing age. This increase was more marked in the toes than in the fingers and was greater in the diabetic than in the nondiabetic subjects. The data revealed a more rapid increase in vibratory threshold among the diabetic subjects during the earlier decades during the later years of life the threshold of the nondiabetic subjects rapidly approached the level of the diabetic subject.

This study provides evidence for the concept that the process of aging is more rapid or of greater initial intensity in the patient with diabetes mellitus than in the nondiabetic subject.

## 65. MECHANISM OF HYPERTHERMIA NOT DUE TO INFECTION

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Fever usually indicates infection but also occurs when an individual cannot eliminate heat produced by his body metabolism, a principle utilized in fever production by the various cabinets.

In the past few years a number of patients have been observed with fever not easily ascribed to infection. Records of nine such patients permitted study of the rectal temperature state of the skin surface, pulse rate and rhythm, blood pressure and environmental conditions. Six developed fever ranging from 104° to 107.8° F during hot humid weather and three developed fever ranging from 102.8° to 106° F during moderate weather. Six were postoperative and 3 were medical patients.

Failure of transport of heat from the interior to the body surface with consequent heat retention was indicated in five of the postoperative and two of the medical patients by a cold skin and a high rectal temperature. In each case a circulatory factor was present: auricular fibrillation in two, excessive tachycardia in two, severe hemorrhage in one, shock following myocardial infarction in one, and circulatory collapse in acute liver necrosis in one.

Failure to eliminate heat from a hot skin was present in one patient with ichthyosis who was virtually unable to sweat and in whom anesthesia, surgery, and excessive bed covering combined with very hot weather to produce a rectal temperature of 105° F. An overdose of barbiturate led to hyperthermia of 104.6° F in one patient found unconscious in a hot humid room. Dehydration, pulmonary edema, and anoxia plus a disturbance of the heat regulatory mechanism were contributory factors.

In each patient several factors were operative but the common factor was the inability to lose heat as rapidly as it was produced, usually due to inadequate circulation. A hot humid environment was often the precipitating factor since this interfered with heat loss. The load on the cardiovascular system was increased by heat retention and attendant tachycardia. This led to excessive tachycardia or auricular fibrillation with less efficient circulation and further heat retention.

From the study of these patients with severe hyperthermia one is led to consider the factor of heat retention in fevers of lesser degree such as occur in congestive heart failure, shock, myocardial infarction, etc. Measures aimed at facilitating heat loss at the inception of fever may prevent the serious effects of hyperthermia.

## 69 THE EFFECT OF HIGH VAGUS SECTION UPON THE CLINICAL PHYSIOLOGY OF THE BRONCHI

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Other than the occurrence of substernal pain in patients having acute bronchitis, little is known concerning pain of bronchial origin. Consequently, an evaluation of the quality as well as the referral of pain of bronchial origin was made in patients by direct stimulation of the bronchial mucosa, using an electrode introduced through a bronchoscope. Following recovery from the bronchoscopy, the patients described the sensation as a moderately intense aching pain, which was felt in the homolateral anterior chest or the homolateral anterior cervical region. In a series of twenty-five patients thus investigated, the referral of pain was consistent and symmetrical.

Patients found to have inoperable bronchogenic carcinoma at exploratory thoracotomy afforded an opportunity to evaluate the effect of vagotomy on the clinical physiology of the bronchi. Accordingly, in these patients the vagus nerve was transected above the pulmonary plexus, yet below the origin of the recurrent laryngeal nerve, with the following results:

(1) The *cough reflex* arising from the homolateral bronchial tree was abolished in all instances. (2) In the majority of cases *pain* of bronchial origin was abolished on the homolateral side. In the remaining few, there was referral of pain to the contralateral anterior cervical region. (3) No effect on the physiologic respiratory change in *bronchial caliber* was noted, nor was any effect observed on *bronchial motility*. (4) On bronchoscopic observation no subsequent changes were noted in the amount nor in the consistency of *bronchial secretions*, likewise, postbronchography roentgenograms revealed no impairment of Lapidol clearance of the tracheobronchial tree. (5) In a limited number of cases, electrocardiographic tracings were unaffected by compression of the vagus nerve or its transection or by electric stimulation of either the proximal or distal ends of the transected vagus.

## 70 PYRIDOXINE DEFICIENCY IN HUMAN BEINGS INDUCED WITH DESOXYPYRIDOXINE

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Since 1934, when Gyorgy announced the existence of a new vitamin for the rat, which when synthesized was called pyridoxine, numerous reports have appeared clearly defining deficiency states in various laboratory animals. These have included seborrhea-like dermatitis, muscular weakness, decrease in lymphoid tissues, and decrease in circulating antibodies in the rat, hypochromic anemia in the dog, anemia and central nervous system lesions in swine, and lymphocytopenia and absolute increase in neutrophils in the monkey. There has never been a clear-cut demonstration that pyridoxine is essential to human beings and a pyridoxine deficiency state in man is unknown.

With the recent advent of metabolite inhibitors, a new tool has been placed in the hand of the investigator to study deficiency states. Such antagonistic activity toward pyridoxine has been demonstrated for desoxy pyridoxine in the chick. During pilot experiments to determine the toxicity of desoxypyridoxine in man, we observed a deficiency syndrome precipitated

by a vitamin B complex poor diet and desoxy pyridoxime which responded specifically to pyridoxime. These early experiments have been extended and are the basis of this report.

Eight patients suffering from various chronic illnesses were given 60-150 mg of desoxy pyridoxime intramuscularly daily while they were maintained on a diet low in vitamins of the B complex. Hematologic studies were done before and at biweekly intervals after the institution of the experimental regime. Urine assays for pyridoxime and thiamin riboflavin and N<sup>1</sup> methyl nicotinamide were done at weekly intervals in most instances. When the deficiency state developed the patient was placed on a vitamin B complex mixture devoid of pyridoxime and later on pyridoxime alone. The desoxy pyridoxime and diet were continued throughout the entire experimental period.

Seborrhea like skin lesions developed about the eyes, nose, and mouth within two to three weeks in seven of the eight patients. In three of the seven the lesions were quite severe. Half of the patients developed erosions in and around the mouth resembling cheilosis of riboflavin deficiency and glossitis resembling morphologically that seen in iron deficiency. One patient developed severe systemic symptoms. A mild but definite lymphocytopenia appeared in seven of the eight patients. No memory occurred. These manifestations were unchanged when the vitamin B complex mixture devoid of pyridoxime was given but responded in forty eight to seventy two hours to the parenteral administration of 100 to 200 mg of pyridoxime.

The authors feel that these lesions constitute at least one part of the human syndrome of acute pyridoxime deprivation.

## 71 PANCREATIC DYSFUNCTION AND LIVER DISEASE

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Advances have been made in the knowledge of the etiology and pathogenesis of liver and pancreatic diseases. Recent animal studies by Chalkoff, Best, Gillman, Ivy, and others have shown that fatty deposition in the liver or fibrosis may follow inflammatory changes in the pancreas, pancreatic duct ligation, and pancreatectomy in dogs maintained on a diet of lean meat and insulin. The hepatic changes could be prevented by the feeding of pancreatic tissue and lipotropic agents.

Experimentally there appears to be sufficient evidence to indicate that disturbance in pancreatic function may lead to serious liver disease.

Hepatic changes have been demonstrated to accompany certain pancreatic diseases such as diabetes mellitus and acute pancreatitis. However, pancreatic dysfunction as a cause of serious permanent liver damage has largely been overlooked.

The use of frequent serum diastase determinations and the prostigmine diastase test has increased the accuracy and incidence in the diagnosis of pancreatic dysfunction and has enabled us to detect abnormalities of the pancreas when diseases of other organs in the upper abdomen may be also present.

We wish to report the case histories and laboratory data of five patients who had pancreatic disease as shown by the clinical history, elevated random diastase determinations, and abnormal prostigmine diastase tests and who also had hepatic disease. These diagnoses were confirmed by surgical exploration and biopsy.

The pathology in the liver ranged from periportal round cell infiltration, similar to that seen in cholangiolytic hepatitis, to the marked fibrosis of advanced hepatic cirrhosis.

In none of the cases could the usual etiological agents of liver disease such as toxins, infection, or malnutrition be elicited. The elevated serum diastase values which occurred at intervals in these patients as well as the abnormal prostigmine diastase test are at variance with the low serum diastase values usually found in patients with damaged livers. These findings and the experimental work of Chaikoff and others, which indicate that removal or damage of the pancreas in dogs and rats will result in fatty infiltration of the liver, as well as inflammatory changes and various types of fibrosis, lead us to suspect that pancreatic dysfunction may at times play an important etiological role in the pathogenesis of liver disease.

## 72 CHANGES OBSERVED FOLLOWING THE EXPERIMENTAL INFUSION OF THE DIURETIC SODIUM SULFATE

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(INTRODUCED BY N. C. GIBBERT, M.D.)

The diuretic ability of sodium sulfate was established some sixty years ago in experimental animals. It was first administered parenterally to human subjects in 1934. No adverse effects were reported in normal subjects receiving moderate doses, and when given to patients with diminished urine outputs, it appeared to be nontoxic. Matland encouraged its use for anuria resulting from crush injuries during the "blitz" in England, but it was not widely adopted. Recently the Council on Pharmacy and Chemistry of the American Medical Association granted its approval for the parenteral use of sodium sulfate as a diuretic in the treatment of oliguria or anuria secondary to the crush syndrome, burns, transfusion reactions, toxic hemolysis, and obstructive lesions of the genitourinary tract.

The author observed the use of this diuretic in four patients. In two with cardiac edema, one responded well and the other expired suddenly several hours after 1 liter of isotonic sodium sulfate was administered. Two patients with anuria resulting from hemolytic reactions consequent to receiving improperly typed blood expired without diuresing. Unusually high blood sulfate levels were found following the use of sodium sulfate.

The foregoing clinical observations prompted further experimental study of this drug. Healthy female dogs were anesthetized, after which they were infused with isotonic sodium sulfate. A control group of animals was studied using isotonic sodium chloride. The infusions were maintained for a period of five hours. At hourly intervals arterial blood samples were obtained and subjected to a complete electrolyte and acid-base study. Urine was collected quantitatively during the same intervals and studied similarly. Electrocardiographic tracings were made initially and at the end of each hour. At the end of the five-hour infusion the animals were sacrificed and tissue biopsies obtained.

It was found that a true acidosis developed following sodium sulfate infusion and that this acidosis could be mitigated by the proper use of buffers in the infusion media. Histologic changes were present in the kidneys of those animals infused with sodium sulfate and absent in the control group. Electrocardiographic changes were also demonstrated during the sodium sulfate infusion.

It is concluded that the indiscriminate use of sodium sulfate as a diuretic in cases of anuria is dangerous. By utilizing a buffered solution with the sodium sulfate, it is thought that some of this danger may be eliminated.

### 73 CARDIOVASCULAR CHANGES FOLLOWING THE EXPERIMENTAL ADMINISTRATION OF BARIUM CHLORIDE

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During recent experiments with various heparin preparations it was found that the sodium salts of heparin increased the coronary flow volume while the barium salts of heparin did not show this effect. In earlier work in coronary flow studies we gained the impression that the soluble barium salts acted as a coronary constricting agent. We therefore decided to study the action of this drug on the cardiovascular system in detail.

Healthy mongrel dogs ranging in weight from 10 to 12 kilograms were given barium chloride orally and parenterally in a conscious state. Electrocardiographic and plethysmographic tracings were made. Blood pressures were recorded. The effect on coronary flow was determined in anesthetized animals by cannulating the coronary sinus and also by studies on the empty beating heart.

Barium chloride administration was followed (a) by electrocardiographic changes such as seen in coronary insufficiency and (b) by a variety of plethysmographic changes. A hypertensive effect was produced with a slowing of the heart rate. The plethysmographic tracings indicated a generalized vasoconstrictor effect. Coronary flow was markedly decreased in the experiments conducted.

There appeared to be a difference in tolerance of the drug in various animals. Some would suddenly expire with convulsions and electrocardiographic tracings in these animals indicated that death was caused by coronary insufficiency. Autopsies made immediately after death showed grossly areas which were similar in appearance to those found immediately following ligation of a coronary artery. All of these toxic effects of barium chloride could be delayed or prevented by treating the animal previously or during the experiment with aminophyllin or papaverine.

It has been shown by others that barium chloride is precipitated within a matter of several minutes by the sulfate ions normally present in the blood stream. Therefore it was difficult to explain the prolonged effects that barium chloride administration sometimes exhibits. Because of this paradox colloidal barium sulfate preparations were made and given parenterally. Similar electrocardiographic changes were obtained with this molecular preparation as with the ionized salts.

From these experiments we conclude that the clinical use of barium chloride in the Stokes Adams syndrome or for any other purpose is dangerous and unpredictable.

### 74 KIDNEY EXCRETION DURING AND AFTER HEMOGLOBINEMIA

A METHOD FOR PRODUCTION OF HEMOGLOBINEMIA BY HIGH SONIC VIBRATION

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Our goal in this work was to obtain an experimental animal (dog) with complete suppression of kidney excretion due to hemoglobinemia; the animal to remain alive and die in uremia from five to ten days after the onset of hemoglobinemia. Acute and chronic experiments were done on large healthy male dogs.

Acute experiments were performed using the following methods for producing hemolysis: thermal trauma, intravenous injection of distilled water, intravenous injection of hemoglobin solution, intravenous injection of hemolytic agents, heating and cooling blood, and, finally, use of high frequency sonic vibrations. The latter method proved to be the most useful, and it was employed throughout most experiments. Blood pressure, urine excretion, blood studies, and survival time were studied. For production of the same hemolysis, a magnetostriiction oscillator was used that produced high energy by giving off vibrations in the region of high sonic frequency. The magneto oscillating rod was placed into a glass cup with two side cannulas which were connected to blood vessels. The blood was hemolyzed as it flowed past the rod. An extremely high degree of hemoglobinemia could be obtained, the highest value was 11,125 mg per cent of plasma hemoglobin.

In well-hydrated animals with no hemoconcentration, hemoglobinemia may produce complete suppression of kidney excretion during the period of hemolysis. Following this the animal may develop diuresis when plasma hemoglobin is below 5,000 mg per cent, oliguria when plasma hemoglobin is between 5,000 and 8,000 mg per cent, or anuria when plasma hemoglobin is above 8,000 mg per cent. In an animal with moderate reduction of blood pressure, together with a progressing hemoconcentration, the production of hemoglobinemia with levels above 2,500 mg per cent resulted in anuria.

The duration of the period in which hemolysis was produced varied between ten and sixty minutes. It is felt that, for the study of experimental anuria, rapid hemolysis must be used, in order to obtain comparable experimental animals.

## 75 CARDIAC FACTORS IN 'NEUROGENIC' PULMONARY EDEMA

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Experimental evidence indicates that Starling's principles of fluid balance are applicable to the lung. Pulmonary edema follows either a diminution of blood osmotic tension or an increase in capillary hydrostatic pressure. Furthermore, increased pulmonary hydrostatic pressure, sufficient to provoke edema, results when cardiac overload is unequally thrown upon the left ventricle. It is likewise possible that left ventricular overload may be produced by "neurogenic" factors. Increased intracranial pressure or vagal stimulation are reported to cause pulmonary congestion and edema through cardiovascular responses extraneous to the lungs.

In open-chest preparations of dogs, pulmonary and systemic arterial and venous pressures were simultaneously recorded. The administration of epinephrine (0.25 to 1.0 mg intravenously) produced the usual striking rise of arterial pressure and marked bradycardia. Cardiac dilatation and elevation of pulmonary venous and arterial tensions occurred, with congestion and edema of the lungs. Conversely, when bilateral faradic vagal stimulation was applied, the resulting bradycardia was accompanied by a fall of systemic arterial pressure, pulmonary congestion and edema did not occur. It is suggested that sufficient elevation of systemic arterial pressure, together with critical bradycardia (reduced minute volume output), from epinephrine, may overload the left ventricle if there is continued competence of the right ventricle and optimum venous return. The experiments suggest that neurogenic influences may alter the pulmonary vascular dynamics through adjustments that lead to impaired left ventricular function.

## 76 DIABETES DETECTION

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(INTRODUCED BY MATTHEW HARGROVE, MD)

Diabetes detection is becoming a problem of increasing importance. Early diagnosis may be the only way one can hope to modify or possibly cure the disease. This is a preliminary report on a diabetes detection survey started in September 1948 which is still being carried on at an industrial plant in Milwaukee, Wis. To date 180 patients have been tested, 69 female and 112 male, their ages ranging from 18 to 75 years. The patients are an unselected group who come to the medical department for routine physical examinations or come with minor ailments. No patient with a definite history of diabetes or symptoms suggestive of diabetes is included in the present report. The individual besides having routine laboratory examinations was given 100 Gm of glucose on a fasting stomach and a blood sugar determination two hours after the ingestion of the glucose. The blood sugar determinations were done by the Folin Wu method; recently the Somogyi method has been used in addition.

If the blood sugar report was 130 mg or over the patients were told to eat a normal diet during the next three days and warned against missing meals or fasting. After that period a glucose tolerance test was done. In those patients who fulfilled the following criteria: presence of sugar in the urine together with peak level above 170 mg per cent (Folin Wu) after the ingestion of glucose a diagnosis of diabetes was made. Eleven cases fell in this group.

Five patients were diagnosed as having a prediabetic state because these patients demonstrated diabetic glucose tolerance curves but did not have sugar in the urine. A diabetic curve is one with a peak level above 170 mg per cent and a level above 130 mg per cent two hours after ingestion of 100 Gm of glucose. In these five patients the peak levels ranged from 196 to 216 and none had a return of BS to below 132 in two hours. Two patients were classed as potential diabetic patients. They had peak levels of 225 and 187 respectively, and two hour levels of 108 and 114 with no sugar in the urine.

On the basis of this survey eleven patients or 51 per cent had definite diabetes as proved by an abnormal glucose tolerance and sugar in the urine. Five patients (28 per cent) had abnormal glucose tolerance curves but had no sugar in the urine and two (11 per cent) were classified as potential diabetic patients. None of these patients have symptoms of diabetes. Some have had several determinations during the year and continue to show the same abnormal tolerance curves. Only seven of these patients have fasting blood sugars above 120 mg per cent, the highest being 151 mg per cent.

If one considers the first two groups collectively approximately 79 per cent of the patients will have diabetes. It is felt that screening methods for diabetes will uncover a large number of diabetic patients but many will still be missed unless more accurate testing is done.

# 77 INTERPRETATION OF THE RESULTS OF THE FLOCCULATION TESTS ON BASIS OF BIOPSY FINDINGS AND PROTEIN PARTITION

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The diagnostic significance of the flocculation tests in hepatobiliary diseases is not fully understood despite their established practical value. Therefore, in 187 cases with various liver diseases (226 series of determinations), the results of cephalin flocculation (CF), thymol turbidity (TT), zinc sulfate turbidity (ZST), and the recently described gamma globulin turbidity (GGT) were compared with those of chemical and electrophoretic partition of serum proteins into albumin and the various globulins and, where available, with the findings in liver biopsy specimens. This correlation indicated the following factors as influencing the results of the flocculation tests:

- 1 Reduction of serum albumin, mostly as result of liver cell damage. This increased all flocculation tests except the GGT.
  - 2 Elevation of gamma globulins in hepatic disorders mostly related to proliferation of Kupfer cells and other mesenchymal cells and histologically recognized by appearance of cytoplasmic ribonucleic acid. This is possibly an expression of antibody formation or of stimulation by liver cell breakdown products. This factor tends to elevate all flocculation tests. It also explains the elevation of GGT and ZST in recovering viral hepatitis, being more marked with tendency for cirrhosis formation.
  - 3 Depression of gamma globulin formation in severe jaundice as result of intra- or extrahepatic biliary obstruction, apparently related to excessive bile pigment inhibition of the Kupfer cells.
  - 4 Depression of ZST but also CF and TT (despite elevation of the gamma globulins) by a humoral factor occurring in obstructive jaundice (probably of biliary nature) and occasionally in cirrhosis without jaundice (so far of unknown character).
  - 5 A factor probably resulting from qualitative changes of serum albumin producing elevation of CF and TT, not explained by changes in the albumin/gamma globulin ratio.
  - 6 Lack of a TT enhancing factor, possibly of lipid character but not related to total lipid or cholesterol concentration. This accounts for the often normal or only slightly increased TT in alcoholic cirrhosis.
- Interplay of these factors produces diagnostic patterns of the flocculation tests in most conditions. Only in toxic hepatitis was the pattern not characteristic. The third and fourth factors account for the normal flocculation tests in extrahepatic biliary obstruction and occasionally in hepatitis with intrahepatic obstruction, both even in presence of severe liver damage. Superimposed bacterial infection in extrahepatic obstruction may raise GGT, CF, and TT without necessarily elevating ZST. Understanding of these factors and the performance of all four flocculation tests increases their diagnostic value.



## 78 A PRELIMINARY REPORT ON THE STUDY OF MYOCARDIAL INFARCTION BY AURICULAR CATHETERIZATION

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In a preliminary experience initiating a study of the dynamic effects of acute myocardial infarction six patients were studied by the right auricular catheterization technique. All patients had definite evidence of the onset of an acute infarct both from clinical and electrocardiographic studies ten to seventeen days prior to catheterization. Cardiac rhythm was normal and congestive failure absent. This postinfarction period was selected as the period of initial study because of the theoretical hazards accompanying catheterization immediately following an acute infarct. After sufficient data and experience have been acquired in the study of this period observation will be extended to the more immediate postinfarction period.

In nine control patients hospitalized for a similar period of time with diseases other than cardiac disorders cardiac indices ranged from 28 to 38 liters per minute per square meter of body surface.

No difficulties or complications have been encountered during or after catheterization. Basal oxygen consumption and right auricular pressures during the procedure were within normal limits in all patients.

In three patients cardiac output, arterial blood pressure and heart work were within normal limits. In the remaining three patients all of whom had low blood pressures work was reduced approximately 30 per cent below the lower normal range of the control values.

One patient was catheterized on the tenth and again on the thirtieth post infarction day. At the first catheterization values for output and work were low. Following the usual clinical improvement during the following three weeks, blood pressure and work rose but cardiac output remained unchanged.

These studies are of a preliminary nature and no conclusions relative to factors leading to reduced output or maintenance of a normal output following infarction can be drawn. In the present small series of cases there was no correlation between the apparent size of the infarct determined from the electrocardiogram and the level of cardiac output.

## 79 TWO RARE CASES OF CONGENITAL MALFORMATION OF THE HEART OF THE CYANOTIC GROUP RIGHT HEART CATHETERIZATION AND ANGIOCARDIOGRAPHIC STUDIES

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Two cases of rare anomalies of the cyanotic group which must be differentiated from the tetralogy of Fallot are presented.

The first case is that of a girl  $5\frac{1}{2}$  years of age with symptoms of mild dyspnea and cyanosis on exertion. Cardiac catheterization and angiocardiology demonstrate a complex malformation consisting of (a) a single ventricle, (b) a pseudotruncus arteriosus with probable hypoplasia of the right pulmonary artery, and (c) aberrant venous coronary drainage directly

into the common ventricle. Data and pressure tracings are presented. Differential diagnosis and certain hemodynamic factors, particularly those which may disturb the balance between the systemic and pulmonary circuits, are discussed.

The second case is that of a 4-year-old girl with persistent cyanosis. Data obtained by right heart catheterization reveal (a) transposition of great vessels, (b) intraventricular septal defect, and (c) patent ductus arteriosus. The problem of differential diagnosis in this case between the Eisenmenger complex and Taussig's heart, consisting of transposition of the aorta and overriding pulmonary artery, is outlined and the dynamics are discussed.

## 80 STUDIES ON THE SPREAD OF EXCITATION THROUGH THE VENTRICULAR MYOCARDIUM

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Direct lead electrocardiograms have been recorded on isolated perfused dogs' hearts subjected to certain injuries designed to influence the spread of the excitatory process through the ventricular myocardium. These injuries were produced by the introduction into the left or right ventricular cavity of solutions or crystals of potassium chloride, silver nitrate, phenol and cocaine.

Injuries sufficiently severe to be attended by the electrocardiographic pictures of bundle branch block did not evoke any specific effect which might be ascribed to a bundle branch block consequent to destruction of Purkinje fibers. As complexes typical of bundle branch block developed in epicardial leads, the QRS complex in leads from the ventricular cavity into which the traumatizing agent had been introduced assumed an essentially monophasic character, with an R wave very similar to that recorded simultaneously from a contiguous epicardial lead. Apparently, endocardial and epicardial electrodes bore a similar spatial relationship to the spread of excitation through the intervening ventricular wall.

In an attempt to account for these findings, the following hypothesis was proposed. The speed at which the excitatory process spreads through a certain segment of the myocardial syncytium depends upon the orientation of the fibers in that segment to each other and to the point of origin of excitation. Spread down a strip of fibers of which the long axis is parallel to the long axis of the strip is rapid as compared with spread down a strip in which the fibers run at a right angle to the long axis of the strip. Endocardial activation is rapid not because of the presence of Purkinje fibers, but because the subendocardial bands of myocardium form a network through which excitation can move rapidly along the long axis of the fibers. Spread across the septum in bundle branch block and across the free wall of the left ventricle in the normally activated heart is slow because excitation is moving through fibers, the long axis of which is perpendicular to the advancing wave of excitation.

Testing of this hypothesis was undertaken by isolation of a segment of the ventricular wall, except for a limited attachment at one end of the strip. The spread of excitation down this segment was studied in relation to the disposition of the fibers constituting the strip. The presence of a band of fibers, the long axis of which is parallel to the long axis of the strip, appears to be essential to rapid excitation of the segment. Whether these fibers are epicardial or endocardial makes no difference.

## 81 THE PATHOLOGIC PHYSIOLOGY OF MEGA ESOPHAGUS

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The actual underlying physiopathology of mega esophagus is still a matter of uncertainty, in spite of many theories set forth. Because methods of determining the functional behavior of various parts of the esophagus have heretofore been so equivocal and inadequate we have felt that esophageal motility studies by the balloon and kymograph method might aid in solving this problem. We have made extensive esophageal motility studies under varying conditions of three normal persons, three patients with achalasia and five patients with organic obstructive lesions of the esophagus.

The three normal persons served as controls. They were studied under varying conditions over many hours. These studies show that organized frequent contractions of good amplitude are related normally with powerful peristaltic waves and an excellent deglutition effect as revealed by a strong pull on the Linhorn tube attached to the balloon. They play an outstanding role in Magendie's third stage of swallowing.

Information obtained by fluoroscopy and x-ray film studies during barium swallow and by observed clinical effects on the inflated balloon which acted as a bolus of food was complementary to that obtained from the esophageal motility studies. The data considered together gave adequate conceptions of the action of the musculature of the esophagus. These data indicated that the intrinsic motility of mega esophagus varies greatly, being almost always irregular, uncoordinated, hyporesponsive to stimuli and not completely effective. In fact the intrinsic motility at times completely failed and in one patient was entirely absent indicating total motor paralysis. This differs from that of the organic obstructive lesions of the esophagus in which there usually occurs a normal motility in the segment proximal to the lesion but rarely a hypermotility or even a hypomotility depending upon the degree of obstruction and the stage and complications of the organic disease. Achalasia of the esophagus also differs from true esophagospasm in which there usually occurs a hypermotility and hyperirritability.

These data indicate that the disease in idiopathic mega esophagus is not localized necessarily to the cardiac end of the esophagus as is commonly believed. In our experience functional abnormalities often involve the entire length of the esophagus. These newer observations offer further evidence for and are compatible with the general theory of achalasia as an underlying mechanism of cause of idiopathic mega esophagus.

A discussion will be presented of the effects of atropine and sympatholytic and parasympathomimetic drugs upon the disturbed peristalsis. The clinical implications of these data will be set forth.

## 82 THE PROTHROMBIN ACTIVITY OF HUMAN BLOOD

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In 1943 when the labile factor was discovered it was noted that the addition of a small amount of depiothrombinized rabbit plasma which has a high concentration of labile factor to stored plasma reduced the prothrombin time markedly below normal. Recently it was found that if blood was carefully collected and stored in silicone coated glassware the prothrombin activity decreased on storage but the addition of depiothrombinized rabbit plasma that is excess labile factor reduced the prothrombin time only to the

level of fresh plasma. By means of the direct method of determining prothrombin by adsorption with tricalcium phosphate and elution with sodium citrate, it was further found that plasma stored in glass twenty-four hours or longer contained a much greater quantity of prothrombin than fresh or stored plasma kept in a silicone-coated container.

Obviously prothrombin activity increases in plasma stored in glass. This is masked by the decrease in labile factor and only becomes evident when labile factor is readded. In a silicone-coated container such an augmentation does not occur during several days of storage. The change in prothrombin activity is explained by postulating that prothrombin in circulating blood exists both in an active and in a precursor state. In contact with a rough surface such as glass the precursor is converted to the active form. In isolated plasma the activation is relatively slow, while in native plasma it occurs in less than an hour. What factor other than the surface of the container is necessary for this conversion to active prothrombin remains undetermined. Platelets, calcium, and thrombin are not essential for this reaction.

Total and free prothrombin and the conversion to the active form are normal in hemophilia and thrombocytopenic purpura. In Dicumarol poisoning both active and total prothrombin are reduced. In one type of congenital hypoprothrombinemia both the active and total prothrombin are below normal, in a second type the active is low but the total may be normal.

In summary, prothrombin in human blood occurs partly in an active form and partly in a precursor state. Prothrombin activity as measured by the prothrombin time is dependent upon the concentrations of (1) active prothrombin (not total), (2) labile factor, and (3) bound calcium. Whether the inactive prothrombin acts as a reserve or becomes activated during standing is not known.

### 83 THE EOSINOPHIL RESPONSE IN ADRENOCORTICOTROPIC HORMONE (ACTH) THERAPY

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Ten patients with diagnoses of rheumatoid arthritis, ulcerative colitis, bronchial asthma, and other allergic disturbances were observed prior to, during, and, in some instances, following the cessation of therapy with ACTH. Aimed by means of serial direct counting chamber eosinophil determinations of the peripheral blood with propylene glycol-phloxine stain diluting fluid as previously described by one of us (T. G. R.).

An average of eleven blood eosinophil determinations was made per patient prior to starting ACTH therapy. The average number of circulating eosinophils was found to be 472, the range varying from a minimum of 55 cells to a maximum of 2,354 cells per cubic millimeter of blood.

Eosinophils disappeared from the peripheral circulation following the intramuscular administration of ACTH in all instances, confirming the observations of Hills, Forsham, and Finch. An absence of circulating eosinophils was first noted in one case at six hours and in another at seventy-two hours, with an average of twenty-eight hours. The dosage of ACTH ranged from 40 to 200 mg per day. The total quantity required for this eosinophil response varied from 15 mg in one individual to 400 in another, averaging 112 mg per twenty-four hours.

One patient was treated with 100 mg daily for seventy-eight hours, two for fifty-four hours, receiving a total of 325 and 225 mg ACTH, the eosinophils

returned to pretreatment levels three four and six days respectively following cessation of therapy. One patient treated at a level of 200 mg daily for thirteen days developed an elevation in eosinophils to thrice his pretreatment average level coincident with a reduction of his dose to 150 mg.

Pretreatment examinations of the nasal secretions and sputa in three patients revealed high levels of eosinophils; these cells disappeared from the nasal and bronchial secretions between twelve and twenty four hours after the onset of ACTH therapy.

#### 84 ALLERGIC REACTIONS FOLLOWING THE INTRAVENOUS INJECTION OF CORN SUGAR (DEXTROROSE OR GLUCOSE)

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Pyrogen contamination has been considered as the major cause of reactions associated with intravenous dextrose therapy. Although the institution of sterile vacuum packed fluid containers and sterile packed disposable recipient sets has largely controlled these factors, disturbing clinical reactions still occur. The fact that these reactions appear in selected individuals suggests that the cause may be due to some peculiarity of the recipient.

So completely have the terms dextrose and glucose been dissociated from their source material that it is not generally realized that all dextrose or glucose is prepared from the simple hydrolysis of corn starch. The fact that the ingestion of corn starch and corn sugar (dextrose or glucose) causes allergic responses in corn sensitive individuals raises the possibility that corn sugar might also act as an allergen when it is injected.

Of several patients previously shown to be highly corn sensitive four were selected for this report. In each case the diagnosis of corn sensitivity was made as a result of the experimental feeding of corn meal gruel and corn sugar after four days of complete corn avoidance. Twenty five cubic centimeters of 5 per cent dextrose were injected slowly intravenously under sterile and pyrogen controlled conditions. In each case severe constitutional reactions occurred which were clinically similar to those observed following the ingestion of corn sugar and corn meal. The control injection of invert sugar of cane origin failed to produce a similar response with the exception of one patient clinically sensitive to both corn and cane. Similarly the control injection of normal saline was without effect.

The importance of these observations cannot be dismissed inasmuch as corn sensitivity and wheat sensitivity are of approximately equal occurrence ranking as the first two foods responsible for the production of chronic food allergy.

Clinical reactions from the intravenous injection of corn sugar occur both in undiagnosed and diagnosed cases of corn sensitivity. More violent reactions may be expected in the corn sensitive individual who has avoided sources of corn in the diet either intentionally or inadvertently for several days prior to the intravenous administration of dextrose or glucose.

## 85 THE DETERMINATION OF THE BASAL METABOLISM BY PERIODIC MAXIMAL EXHALATIONS

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The measurement of the basal metabolism is subject to considerable variation through the natural inability of many subjects to breathe evenly. We have investigated the possibility that the chest position after a voluntarily forced exhalation might be less variable than after a quiet expiration and therefore lead to a more reliable method of estimating the basal oxygen consumption.

One hundred and fifty consecutive subjects of both sexes, with great diversity in age, height, weight, intelligence, and illness, were studied clinically. Their oxygen consumption was measured by the test and standard methods. The test method was a modification of the standard in that every minute the subject was directed to force a maximal exhalation. The first sixty-two subjects were tested by both methods. For the second group of fifty-four subjects a third method was introduced, which modified the test method by continuing for twelve or fourteen minutes and by including early in the test the performance of the maximal breathing capacity. The last group of thirty-four subjects performed the standard and test methods in duplicate. The order in which all tests were done in each series was determined from a prearranged schedule derived from a table of random numbers.

The test procedure does not measurably affect the rate of oxygen consumption. It results in significantly less variation between duplicates. It usually corrects for systematic or irregular deviations in the depth of undirected breathing that commonly lead to either an indeterminate conclusion or to a fallacious inference as to the true rate of oxygen consumption. The uniformity of the end point of forced exhalation is unaffected by such factors as lung disease, congestive heart failure, dysthyroidism, lack of training, or low intelligence of the subject and is less affected by consciousness of breathing than is the standard method.

Therefore this simple modification of the standard method of measuring the basal metabolism allows the rate of oxygen consumption to be estimated in some subjects when the standard method is unreliable, and in all subjects the estimate is usually significantly more precise.

## 86 IDIOPATHIC DILATATION OF THE PULMONARY ARTERY

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Increased interest in the differential diagnosis of congenital heart disease has attracted attention to an unusual disorder characterized by dilatation of the main pulmonary trunk with or without dilatation of the pulmonary arterial branches. This change in the pulmonary artery is unaccompanied by any other structural cardiac or pulmonary disease. It is considered a congenital cardiovascular disorder but its etiology is undetermined. Less than twenty cases which are acceptable by relatively strict criteria have been reported, but there is evidence to suggest that this anomaly is more common than has been assumed.

Five examples of idiopathic dilatation of the pulmonary artery have been observed. In two of them the diagnosis was established at autopsy and in

one of these the diagnosis was made ante mortem. This patient was observed over a period of ten years. The dilated pulmonary arteries were demonstrated angiographically six months before her death. At that time the right, main pulmonary arterial branch was completely occluded by a calcified thrombus. The post mortem examination disclosed a pulmonary trunk which was actually considerably larger in diameter than the heart itself. The three additional patients are living but the clinical picture seems compatible with the diagnosis in all respects.

This disorder seems to predominate in female subjects. It has been observed from childhood to old age. Dyspnea, cough, cyanosis and hemoptysis are common symptoms although the patient often has few complaints until late in his course and the final illness may be very brief. The tolerance for exercise and pregnancy is usually surprisingly good. The major abnormalities disclosed by physical examination are a systolic murmur and a systolic pulsation in the pulmonic area, accentuation of the pulmonic second sound, and often a diastolic murmur along the upper left sternal margin. The abnormally dilated right outflow tract is easily visualized radiographically as well as in angiocardiograms and abnormal pulsations of the pulmonary arteries are common. The electrocardiograms usually show incomplete or complete right bundle branch block or evidence of right ventricular hypertrophy. Recent studies by Courand and associates have indicated that in these cases the systolic pressure within the dilated pulmonary artery is not elevated and in fact is lower than in the right ventricle.

## 87 THE EFFECT OF AUROMYCIN ON UROBILINOGEN FORMATION AND THE FECAL FLORA

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The effect of aureomycin on urobilinogen formation has been studied in a series of fifteen patients. The amount of fecal urobilinogen was determined serially in seven of these single determinations being made in the remainder. Bacteriologic studies of the feces were carried out on eleven samples in four cases. In addition the amount of urobilinogen in the bile obtained by duodenal tube was determined in eight cases.

These studies reveal that following aureomycin and concomitant with a great reduction or practical disappearance of coliform organisms in the feces the urobilinogen of the feces, urine and bile markedly diminishes the values often falling into the range which characterizes complete biliary obstruction. The bile urobilinogen was entirely absent in seven of the eight instances, only a trace being demonstrated in the eighth. This may be contrasted with the uniform presence of small amounts in fifty individuals not receiving aureomycin. The Harrison test for bilirubin applied to the feces often became positive after aureomycin but the amounts did not appear to correspond with the reduction in amount of urobilinogen.

In a case of subacute bacterial endocarditis in which urobilinogen studies were first carried out after one month of continuous aureomycin therapy the feces contained very little bilirubin but gave a fairly intense Ehrlich reaction for urobilinogen. It was possible to demonstrate however that no stercobilinogen was present; polarimetry revealed that the only urobilin represented was dextrorotatory rather than levorotatory. It was believed significant that the bile was now Ehrlich negative indicating that the fecal urobilinogen was not hepatic in origin. The feces at this time contained colon aerogenes inter

mediates but no typical *E coli*. Nonproteolytic clostridia were also present. This combination is of interest since Kammeier and Miller, and, more recently, Baumgartel have ascribed the reduction of bilirubin in the colon to *E coli* and spore-forming anaerobes. In the present study *E coli* was regularly lacking in the feces after aureomycin and it is evidently essential to the reduction of bilirubin to stercobilinogen. It appears, however, that other organisms of aerogenes type are at times able, perhaps in association with clostridia, to effect a partial reduction of bilirubin, either to mesobilirubinogen or a dextrorotatory derivative thereof.

These results strongly support the belief that the urobilinogens of the feces, bile, and urine are wholly enterogenous. In recent years Baumgartel and co-workers have described evidence suggesting that, of the two urobilinogens, mesobilirubinogen is hepatogenous, only stercobilinogen being enterogenous. The present study reveals, however, that aureomycin markedly depresses the total urobilinogen formation and that under its influence urobilinogen completely disappears from the bile. These findings are incompatible with the hepatogenous concept unless one were to assume that aureomycin interferes with liver function, for which there is no evidence.

## 88 PAROXYSMAL MYOHEMOGLOBINURIA WITH FATAL RENAL TUBULAR INJURY

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(INTRODUCED BY C. J. WATSON, M.D.)

A fatal case of paroxysmal myohemoglobinuria in a 23-year-old man is presented.

The history was suggestive of a familial occurrence. Fifteen cases have been described in the literature, in three of which there was a history of familial incidence.

Death was due to renal insufficiency and pulmonary edema. The myohemoglobinuria, which had been observed for the first three days of observation, disappeared six days before death. Myohemoglobinemia was carefully looked for but never demonstrated.

In the present case, the myohemoglobin in the urine was identified and distinguished from ordinary hemoglobin by virtue of its spectral distribution curve. Preliminary study of porphyrin metabolism revealed a moderate increase in fecal coproporphyrin, 60 per cent of which was type III.

Significant histologic changes were found in the striated muscles and kidneys. The uremia and subsequent death were the result of tubular injury, as indicated by the demonstration of hydropic degeneration of the proximal convoluted tubules. Although pigment casts were found, they did not appear to be sufficient in number to have been the cause of the uremia, as has been suggested by others. The finding of these casts supports the concept that the precipitation of pigment is more likely the result of disturbed renal function rather than the cause of it.



## 89 THE CLINICAL AND LABORATORY EFFECTS OF HYPOTONIC INTRAVENOUS INFUSIONS

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We have found hypotonic solutions given by vein effective in furnishing water whenever the customary amounts of sodium chloride and dextrose present in standard isotonic solutions were undesirable or unnecessary. The use of hypotonic solutions has in our experience been attended with good clinical results and has permitted a more exact maintenance of water and electrolyte balance. Hypotonic solutions of dextrose and of sodium chloride were first used in correcting disturbances of the extracellular fluid (pattern disturbances volume losses and plasma water deficits) in diabetes. Further experience has shown that these solutions are equally valuable in the more seriously ill patients who have a large antecedent plasma water deficit or who have a continuing excessive demand for plasma water. For the most part we employed solutions of one half isotonic strength and the three most commonly used solutions had the following composition: (1) 25 Gm of dextrose per liter of distilled water (2) 4.5 Gm of sodium chloride per liter (3) 12.5 Gm of dextrose and 2.25 Gm of sodium chloride per liter.

Thus far our clinical experience embraces more than fifty patients who have received hypotonic solutions in amounts varying from 1000 to 6000 cc in twenty four hours over periods of from one to twenty two days.

Immediate observations on the blood were made in twenty three patients after 1000 cc of a hypotonic solution had been given. Twelve patients received 2.5 per cent dextrose and eleven received 0.45 per cent sodium chloride. The infusion was completed in periods of time varying from thirty to ninety minutes. Determinations of the plasma hemoglobin, total plasma protein, plasma specific gravity, hematocrit, percentage of erythrocytes, and the hemoglobin were made before the intravenous infusion was started, immediately after it was finished, and one hour after it was finished. As in earlier studies made after the prolonged use of large amounts of these solutions, but on blood drawn four to twelve hours after the last intravenous infusion had been given, none of these immediate determinations showed any evidence of harmful degrees of dilution. A transient slight fall in their values shown in most of the determinations made immediately at the end of the intravenous infusion was followed in the majority by a tendency to rise toward the original levels at the end of one hour. The blood showed no plasma hemoglobin except in two patients; in the one in whom the experiment was repeated with the same intravenous solution, no plasma hemoglobin was found.

## 90 STELLATE BLOCK IN THE MANAGEMENT OF NARCOLEPSY AND CATAPLEXY

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The effect on narcolepsy of the injection of procaine into the cervical sympathetic chains in four patients with narcolepsy and cataplexy, one patient with narcolepsy and one patient with hypersomnolence forms the basis of this report. Because of observations on alterations of cerebral function by temporary interruption of sympathetic impulses to the brain it was decided to ob-

serve the effect in narcolepsy. Associated symptoms and signs in these six subjects have been reviewed, and an attempt has been made to correlate these with the response to stellate blocks. Other common causes for excessive drowsiness were excluded by a number of laboratory procedures. An attempt was made to correlate the response of symptoms to stellate blocks and to the administration of Benzedrine and closely allied drugs.

The urinary excretion of gonadotrophic hormone (FSH) prior to and following stellate blocks was studied whenever possible. An electroencephalogram tracing in one subject discloses a very abnormal deep sleep pattern prior to stellate block. Repetition of the electroencephalogram in this same subject after stellate block injection shows a distinct difference, with a tendency toward a more normal pattern.

In general, patients with narcolepsy and cataplexy have their symptoms controlled for a period of time following stellate block injection, but the effect is more favorable upon the narcolepsy than upon the cataplexy. No change in urinary gonadotrophin excretion was seen to follow the procedure.

## 91 A SYNDROME OF HYPERTENSION, OBESITY, MENSTRUAL IRREGULARITIES, AND EVIDENCE OF ADRENAL CORTICAL HYPERFUNCTION

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AND HELEN E. CLARK, M.D. (BY INVITATION), ST. LOUIS, MO

Twenty-four women suffering from arterial hypertension were found to possess certain common clinical and laboratory findings which suggested that they belonged to a previously undescribed symptom-complex. These were (1) relatively sudden onset of obesity occurring at menarche, menopause, after multiple pregnancies or gynecologic operation, (2) obesity of the "central type," with pale striae on thighs and sometimes on upper arms, (3) menstrual irregularities, (4) good or excellent therapeutic response of blood pressure levels to diets low in salt, with reversals when the intake of salt was raised, (5) abnormally low concentrations of sodium and chloride in sweat. Secondary and less common signs and symptoms included an aversion to salty foods and high fluid intakes, unexplained periods of oliguria, inconstant glycosuria or a diabetic type of glucose tolerance curve which was accompanied usually by increased sensitivity of the blood sugar levels to intravenously injected insulin, plasma chloride levels above or at upper limits of normal, hirsutism, a tendency to easy bruising and ecchymoses, a relatively benign type of hypertension with little albuminuria or other signs of renal damage, and sensitivity of the blood pressure to injected desoxycorticosterone acetate or glucoside. In no instance were the cardinal manifestations of Cushing's syndrome present. Because these symptoms, signs, and findings were seen rarely in other types of hypertension and because concentrations of sodium chloride in sweat were normal in most subjects, including obese nonhypertensive women, it is believed that these patients represent a separate pathogenetic entity. The evidence is strongly suggestive that certain functions of the adrenal cortex are specifically disturbed in these patients, but not in other forms of hypertension or obesity.

92 A LONG TERM EVALUATION OF THE THERAPY OF  
PERNICIOUS ANEMIA WITH FOLIC ACIDSTEVEN O SCHWARTZ MD SHERMAN R KAPLAN MD (By INVITATION),  
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Folic acid (pteroylglutamic acid) was introduced in 1945 as a specific therapeutic agent for liver extract principle deficiency diseases. Numerous short term observations quickly confirmed its value. Because the natural history of pernicious anemia is characterized by exacerbations and remissions it was felt that long term studies were necessary in order to critically evaluate any new therapeutic agent.

Early in 1946 ninety eight patients with pernicious anemia were selected for such a study from the Out patient Anemia Clinic of the Cook County Hospital. Their course liver extract requirements neurological status and other characteristics were well established the patients having been followed by the same group of observers and maintained in remission with liver extract for periods ranging from months to years. Folic acid was administered orally in a daily dose of 5 milligrams. The patients were seen at four week intervals for interviews and neurological and hematologic examinations.

The present report summarizes the observation made during the three and a half years the study has been in progress. There have been thirty one neurological and thirty hematologic relapses with eight patients showing a combination of both. In three patients the folic acid was discontinued because of persistent weight loss and/or soreness of the mouth. Twenty nine patients discontinued their therapy for reasons beyond our control. Most of these patients disappeared from observation many however only after insisting that they felt better while on parenteral liver therapy which they wished resumed. Of the ninety eight patients only thirteen have been able to remain on continuous oral folic acid therapy and maintain themselves in remission.

Without exception every hematologic relapse was quickly reversed with a change to parenteral liver therapy. In fourteen patients with neurological relapse however the changes have not been reversible in spite of intensive therapy with liver extract for six months or more. Bone marrow aspirations were performed in five patients at the time of relapse. Megaloblastic changes were demonstrable in all instances.

The original plan envisioned an attempt to increase the dose of folic acid in those patients who showed evidences of relapse but the impression (gained from the current reports of the time) that larger doses of folic acid precipitated neurological relapses faster than small doses deterred us.

93 HEMOPOIETIC CHANGES DURING CHLOROMYCETIN  
ADMINISTRATIONITALO F VOLINI MD STEVEN O SCHWARTZ MD IRVING GREENSPAN MD  
(By INVITATION), LEE EHRLICH MD (By INVITATION) JAMES A GONNER MD  
(By INVITATION) AND OSCAR FELSENFELD MD (By INVITATION)  
CHICAGO ILL

Toxic manifestations of oral administration of chloromycetin have not been reported. Recently we have observed three patients in whom hematologic abnormalities were encountered during chloromycetin therapy.

Two patients with typhoid fever and one with acute brucellosis were treated with oral chloromycetin for periods ranging from nine to nineteen days. The total dose of chloromycetin ranged from 30 to 60 Gm and was given in a uniform dose of 3 Gm per day (0.5 Gm every four hours).

A significant fall in the white blood cell count, attributable to the diminution in the number of granulocytes, could be demonstrated as early as the sixth day of therapy. White cell counts as low as 3,000 and absolute neutrophil counts of 264 were observed. No significant changes in the total number of lymphocytes were demonstrated. A slight drop was seen in the red cell count and the hemoglobin. In one instance this drop was out of proportion to a fall ascribable to the underlying infection.

The marrow was studied in all patients. In the first, a marked granulopenia and left shift in erythropoiesis were present on the eleventh day of therapy. In the second patient the marrow was studied three and thirteen days after the discontinuance of the chloromycetin. On the third day a left shift in granulopoiesis as well as erythropoiesis was seen, with an increase in eosinophils. By the thirteenth day the left shift had disappeared in both series and evidences of marked regeneration accompanied by an extraordinary eosinophilia were seen. In the third patient, the marrow was studied at the beginning of therapy and revealed the changes expected with infection. Eleven days later (two days after discontinuance of therapy) granulopoiesis had markedly diminished and a left shift had occurred in erythropoiesis.

Upon the discontinuance of the chloromycetin there was an immediate spontaneous reversal of the downward trend in the blood values.

In the three patients studied there is evidence that within a few days chloromycetin produces a marrow hypoplasia more marked in the granulocyte series but also involving a maturation arrest of erythroid elements. The peripheral blood simply reflects the more fundamental changes in the marrow. In the therapeutic doses employed in the present series the marrow alterations are reversible.

Both clinical and experimental studies are in progress to verify and extend these findings.

#### 94 EXPERIMENTAL SYPHILIS IN THE RABBIT THE RELATIONSHIP OF METACHROMASIA TO FIBRINOID DEGENERATION OF COLLAGEN AND THE LOCALIZATION OF SPIROCHETES IN THE TESTIS

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Since the earliest studies of experimental syphilitic orchitis there has been recognized, as a characteristic feature of the inflammatory reaction in the testicular stroma, the deposition of a relatively acellular, loosely constructed material resembling Wharton's jelly. The term "mucous (or mucoid) degeneration" of the connective tissue has been applied to this change. The material stains metachromatically and has been found in greatest abundance in the tunica albuginea, particularly around blood vessels.

We have confirmed the preceding observations in a study of syphilitic orchitis in the rabbit using the Nichols' strain of *T. pallidum*. In addition, we have found that previous incubation of the sectioned tissue in hyaluronidase (Schering) abolishes the capacity for metachromatic staining with thionin and with toluidine blue. No metachromasia is found in the stroma of the nor-

mal testis. Focal fibrinoid degeneration of collagen has been observed in the metachromatic areas and the highest concentration of spirochetes has been noted in and around these sites.

It may be concluded that the loosely constructed relatively acellular material which appears in large amounts in the stroma of the syphilitic rabbit testis and which stains metachromatically contains hyaluronic acid or a closely related substance. It would appear that the sites of localization of the metachromatically staining material, the fibrinoid degeneration of collagen and the highest concentrations of spirochetes are intimately related spatially. A sequential study of syphilitic orchitis in the rabbit is now in progress to investigate the possible causal or temporal relationships between the multiplication of spirochetes, the development of metachromasia and the appearance of fibrinoid degeneration of collagen.

## 95. CONTINUOUS DICUMAROL PROPHYLAXIS IN CORONARY DISEASE

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It seemed reasonable to suppose that an agent which would prolong coagulation time and have a deterrent effect on blood sludging might be of value in preventing thrombosis in chronic arterial disease. Accordingly twenty-one patients have been given Dicumarol continuously for periods of three weeks to three years. Of these eighteen had clinical and electrocardiographic evidence of coronary thrombosis with myocardial infarction, two had coronary sclerosis with angina of effort and one had cerebral thrombosis with left hemiparesis.

After initial stabilization prothrombin times were determined weekly by Quick's method and Dicumarol was prescribed in daily doses sufficient to maintain prothrombin time between two and two and a half times normal control values. Individual requirement varied from 50 mg. every other day to 150 mg. daily.

Five patients showing marked sensitivity to Dicumarol and unable to take as much as 50 mg. daily without alarming fluctuation in prothrombin time were given menadione bisulfite 2 mg. daily. These showed increase in tolerance and prothrombin times thereafter were more readily controlled.

All patients were advised of the risks involved and instructed in the symptoms and signs of overdosage.

No disastrous toxic effects have been encountered. Transient hematuria occurred in three patients, rectal bleeding from hemorrhoids which had bled prior to therapy occurred in one patient, cutaneous ecchymoses occurred in three patients, epistaxis occurred in one patient. One man with previous history of bleeding duodenal ulcer bled following extraordinary dietary indiscretion although his prothrombin time was within the desired range. The drug was discontinued and coronary thrombosis recurred within ten days. Dicumarol was then resumed and he has been well and at work for the past ten months.

There have been no deaths in the series. Chest pain recurred in two patients when the drug was discontinued because of toxic effect and ceased when Dicumarol was resumed. Anginal attacks have ceased entirely in eight patients and have been markedly reduced in frequency and severity in two. All of the patients have resumed their former activities.

Electrocardiograms in five cases have shown a striking reversion toward normal.

It is surprising and worthy of note that all patients have followed instructions carefully and have expressed the desire to continue under treatment indefinitely in spite of the inconvenience involved.

Results seem to indicate that continuous and controlled Dicumarol therapy is practicable and may influence favorably the course of disease of the coronary arteries.

## 96 THE USE OF ORAL MERCURHYDRIN COMBINED WITH ASCORBIC ACID IN CARDIAC DECOMPENSATION

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The effect of the oral administration of a tablet containing 60 mg of an inorganic mercurial compound (Mercurhydrin), equivalent to 19.5 mg of mercury, and combined with 100 mg of ascorbic acid has been studied in one hundred patients with congestive heart failure. The patients' ages ranged from 12 to 60 years and the congestive failure was due to valvular, hypertensive, or arteriosclerotic heart disease. From one to three tablets were administered daily.

These tablets were equally effective as one containing 120 mg of Mercurhydrin alone. They were satisfactory in controlling mild congestive failure in approximately one-third of the patients when used as a primary treatment. They were effective in controlling mild to moderate congestive failure in approximately two-thirds of the patients after maximum compensation had been obtained following injectable Mercurhydrin. They were usually ineffective in controlling severe failure when used alone, but frequently of value when used as a supplement to injections. Toxicity, when it occurred, was mild and transient in the large majority of instances.

The studies indicated that the tablets may be used either alone or as a supplement to parenteral therapy to assist in the control of congestive heart failure.

## 97 SMALL BOWEL CHANGES IN AMEBIASIS

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Following an attack of amebiasis, many patients manifest a multiplicity of abdominal symptoms which, though nondescript and not too severe, frequently make them gastrointestinal problems. The frequent finding on cytologic examination of the stool of epithelial cells originating from the small bowel suggested the latter as the possible site of "lesions" leading to the mentioned symptoms. Hence, all patients with chronic amebiasis had x-ray films made of both the small and the large bowel for possible pathologic changes.

Of sixty patients with chronic amebiasis thus examined, twenty-seven showed changes from the normal in the small bowel, particularly the terminal ileum. These changes are not seen as a rule in ulcerative colitis, sprue, and various nutritional deficiencies.

Spasm and edema of the terminal ileum are the changes most often noted. More characteristic, however, are the edematous folds of the terminal ileum which sometimes made the terminal ileum appear almost as wide as the colon.

The nonspecific changes of segmentation and pooling is seen in sprue or the ulcerated involvement of the terminal ileum as seen in ulcerative colitis were not observed in these patients. The motility of the small bowel appeared undisturbed.

The patients demonstrating the described changes in the terminal ileum were in general more difficult to manage clinically especially with regard to control of the cramping abdominal pain.

## 98 PHYSIOLOGIC AND PHARMACOLOGIC STUDIES IN A CASE OF PHEOCHROMOCYTOMA

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AND EUGENE B. FERRIS, M.D. (CINCINNATI, OHIO)

A case of pheochromocytoma subsequently proved at operation and at autopsy, allowed us the opportunity to compare in a single patient the physiologic and pharmacologic specificity of a number of suggested diagnostic procedures. The patient, a woman with persistent hypertension for approximately one year, preceded by at least three years of paroxysmal hypertension, had a blood pressure which ranged from 215/140 to 200/155 mm Hg.

Benzodioxane (933I) produced an immediate fall in pressure to normotensive levels. Dibenamine produced a similar fall which was slower in onset and persisted longer. Tetraethylammonium chloride (TEAC) produced a sharp rise in pressure with failure to return to initial levels for some time; this rise was shown to be abolished by benzodioxane. Diminished sensitivity to the pressor activity of intravenous adrenalin was demonstrated. Histamine and mecholyl each caused a fall in pressure, the rise reported to be characteristic of pheochromocytoma being absent.

The results bear out the specificity of benzodioxane and Dibenamine in counteracting the pressor activity of circulating epinephrine. Response to benzodioxane seems more specific, however, because no depressor effects have been encountered in a series of control hypertensive subjects, whereas such reactions do occur with Dibenamine. Diminished sensitivity to exogenous adrenalin would also appear to have specific meaning, in that tolerance to the effect of this drug appears to develop when it is present in the circulation in large amounts. The pressor response to TEAC would appear humoral in nature, specifically adrenergic in this instance since it was abolished by benzodioxane. The lack of a pressor response in this case to either histamine or mecholyl suggests that these tests are not specific for diagnosis.

The results suggest the specificity of benzodioxane in the diagnosis of pheochromocytoma when the pressure is elevated and the desirability of evaluating the mechanisms of pressor responses to histamine, TEAC or other stimuli by administration of benzodioxane.

## 99 HEART BLOCK FOLLOWING MEDULLARY PERFUSION WITH BACTERIAL TOXINS

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The role of bacterial and viral agents in the production of heart block has been known for many years. Of particular interest has been the occurrence of bradycardia and heart block following acute bacterial infections es-

pecially in the case of diphtheria. The question of whether myocardial damage per se or increased vagal tone or a combination of both is responsible has remained to conjecture, with the preponderance of opinion in support of cardiac damage. No adequately controlled experimental evidence has thus far been presented to note the effect of increased vagal tone in the pathogenesis of heart block.

Six commercially produced bacterial toxins were employed in this study, namely *C. diphtheriae*, *Staph. aureus*, *St. hemolyticus*, *Cl. tetani*, *Cl. welchii*, and *Cl. botulinum*. These toxins were perfused through the turtle brain which was connected with the body of the animal only by means of the vagus nerves.

Heartblock was produced with all of the toxins. The blocks produced by *C. diphtheriae*, *Cl. welchii*, and *Cl. botulinum* could be released by atropine. These results suggest an acetylcholine-like mechanism. The remaining toxins were not affected by atropine and the mechanism for their action is unknown.

## 100 THE TAKATA-ARA REACTION IN THE DIFFERENTIAL DIAGNOSIS OF JAUNDICE

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In the work-up of jaundiced patients the Takata-Ara reaction (TAR) seemed of diagnostic help in certain types of jaundice, hence, an attempt was made to re-evaluate the differential diagnostic significance of the TAR on the basis of recent classification of hepatobiliary diseases.

The TAR was performed in 356 cases and compared with the results of cephalin-cholesterol flocculation, thymol turbidity, and zinc sulfate turbidity tests. The percentage of positive TAR's in the different types of hepatobiliary disease were as follows: Laennec's nonfatty cirrhosis, 87.8, fatty cirrhosis, 35.5, postnecrotic (nonalcoholic) cirrhosis, 100, infectious viral hepatitis, 107, homologous serum hepatitis (HSH), 68.0, toxic hepatitis, 27.6, obstructive jaundice (incomplete, benign), 2.0, and obstructive jaundice (complete, malignant), 20.0.

The TAR may therefore assist not only in differentiating a cirrhosis with jaundice from an acute hepatitis but also from an infectious hepatitis. The TAR is not related to the degree of icterus, and the high incidence of abnormal TAR's in HSH is therefore not an expression of the well-known greater severity of the latter.

Moreover, no parallelism was found between the TAR and the cephalin flocculation and thymol turbidity tests. A moderate parallelism existed between the TAR and the zinc sulfate turbidity, except in infectious hepatitis where the latter is moderately elevated and the TAR is negative, in toxic hepatitis this exception is less marked.

A discrepancy between the TAR and the zinc sulfate turbidity is also apparent during the recovery phase of hepatitis when the zinc sulfate turbidity increases while the TAR (if positive) becomes negative. While, in recovering hepatitis, a rise in the zinc sulfate turbidity and a fall in the cephalin-flocculation and thymol turbidity occurs, a rise in the TAR in addition (or the maintenance of a positive TAR) speaks for the transition from hepatitis to cirrhosis. Similarly, the appearance of positive TAR in a fatty cirrhosis seems to indicate progression to a Laennec's nonfatty cirrhosis.

The TAR may indicate certain phenomena not demonstrable by the other flocculation tests.



# 101 A STUDY OF THE MOVEMENTS AND SOUNDS OF HEART VALVES OF VARIOUS LABORATORY ANIMALS (A MOTION PICTURE AND SOUND RECORDING)

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EDWARD J BALDES, PH D (BY INVITATION), ROCHESTER, MINN

The hearts of various laboratory animals were perfused with oxygenated Ringer Locke solution and were kept beating for various lengths of time. Rather large openings were made in the walls of the auricles. This afforded an excellent view of the entire valves. Colored motion pictures were made of the exact movements of the mitral, tricuspid, aortic, and pulmonary valves. A sound record, an electric record of the sound, and an electrocardiogram were made at the same time the motion picture was made. These records enable one to see the movements of the heart valves, to hear the sounds they produce, to see an electric record of the sounds and to see an electrocardiogram recorded at the same time. The results of this study furnish information that we believe will change some of our previous ideas about the factors that cause the mitral and tricuspid valves to open and close.

# 102 CARDIODYNAMIC AND RENAL CHANGES IN SPONTANEOUS AND NEPHROGENIC HYPERTENSIVE DOGS IN RESPONSE TO TISSUE INJURY

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CHICAGO, ILL

Tissue injury, particularly abscess, produces a sustained fall in blood pressure in some spontaneous and nephrogenic hypertensive dogs. This study was undertaken to investigate the cardiodynamic and renal alterations ensuing during this depressor response.

The following determinations were done before and after various types of tissue injury: (1) renal plasma flow (RPF), (2) glomerular filtration rate (GFR), (3) plasma volume, (4) thiocyanate space, (5) central venous, right atrial, right ventricular and pulmonary artery pressures, (6) blood pressure.

It is concluded from the data that: (1) Spontaneous and nephrogenic hypertensive dogs exhibit the same hemodynamic pattern. The cardiac output, plasma volume, thiocyanate space, and pulmonary artery pressure are all within normal ranges. Both types of hypertension are due to an increase in peripheral resistance. (2) Spontaneous hypertensive dogs all have normal GFR and RPF. In contrast, some nephrogenic hypertensive dogs have normal clearances, others exhibit significantly reduced kidney function. (3) A sustained blood pressure fall can be readily elicited by injection of abscess-inducing substances such as turpentine, indicating that the depressor response is a nonspecific one with respect to presumed renal hypertensive mechanisms. (4) Accompanying the sustained depressor response in both nephrogenic and spontaneous hypertensive dogs is a sustained increase in RPF. GFR does not change significantly. Renal vascular resistance, particularly efferent arteriolar, is reduced. (5) The plasma volume, thiocyanate space, venous pressure, and right heart pressures are not affected by tissue injury. The blood pressure fall is not due to hypovolemia. (6) The cardiac

output is increased during the depressor response to injury. The fall in blood pressure is therefore due to a decreased peripheral resistance. Apparently the decrease in both renal and general peripheral resistance is the resultant of a common train of events elicited by injury. There is no evidence for a cause and effect relationship between them. The blood pressure fall following injury cannot be attributed to "relief of renal ischemia."

### 103 CLINICAL EVALUATION OF A NEW LIPASE PREPARATION

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Many agents are available which have the property of digesting fat, yet there is a need for one which would be capable of hydrolyzing a wider variety of lipids.

It was thought, on the basis of experimental evidence in animals, that lipase A may be helpful in individuals who display an inability to adequately digest all forms of food fat. The new preparation is an enzyme, protein in nature, produced during the growth of a certain type of mold (fungus).

In this study lipase A was used in patients having steatorrhea. Control studies also were made. Each capsule contained 0.4 Gm of the enzyme and each patient was given twenty-four capsules daily.

These individuals were placed on a calculated diet of low fat (50 Gm in twenty-four hours) and evaluated clinically and chemically before and after lipase was given. The chemical determinations were made on the nitrogen, on the total and neutral fat and fatty acids of the stool, the blood was studied as to content of lipase, calcium, nonprotein nitrogen, and total protein, and the urine was studied as to total output of nitrogen. These determinations were made before and after the fat content was changed in the diet.

Comparative study was also made with the use of pancreatin in the same group of patients.

A specific example will demonstrate the efficacy of the new enzyme.

A patient with carcinoma of the pancreas was placed on a 55 Gm fat diet plus adequate protein and carbohydrate. The total fat calculated in the stool before lipase A was administered was 57.0 per cent (dry method). After three capsules of lipase A were given per day for a period of three weeks, the total fat in the stool was reduced to 42.0 per cent. After the diet was increased to 152 Gm fat, the stool fat was elevated to 73.0 per cent. The administration of twelve to twenty-four capsules of lipase decreased the total fat in the stool to 53 per cent and 40 per cent respectively.

Clinically, the patient improved, the stools decreased in number and became more solid, and the cramping in the abdomen subsided. In general, the patient ate better and felt better.

*Conclusion* (1) Lipase A is helpful in steatorrhea. (2) Large quantities of the enzyme are required for optimum results. (3) Pancreatin compares favorably with results obtained with lipase A.

## 104 ANGIOCARDIOGRAPHY

GEORGE C SUTTON, M D GEORGE WENDELL M D, HARRY GRANT, M D, AND  
HAROLD WEDFELL M D CHICAGO ILL

(INTRODUCED BY DON C SUTTON M D)

A comparison is made between angiocardiology by arm vein and direct catheterization of the heart

Direct intracardiac angiocardiology is a procedure possessing few difficulties and of negligible risk. The procedure will give contrast visualization of the heart and great vessels which is routinely superior to that obtained by peripheral injection of a radio opaque material. This is especially true for levoangiocardigrams. Simple inexpensive equipment for changing the films and low power x ray apparatus are more practical for use with intracardiac injection without loss of a very high percentage of diagnostic films. In some cases demanding a high concentration of contrast media and accurate timing of exposures, only intracardiac injection will produce satisfactory angiocardigrams.

### 105 DIVISION OF THE POPLITEAL VEIN IN VALVULAR INSUFFICIENCY OF THE DEEP VENOUS SYSTEM OF THE LOWER EXTREMITIES

GEZA DE TILAKITS M D AND GUSTAV W GRAUPNER, M D (BY INVITATION)  
CHICAGO ILL

From a large number of patients exhibiting valvular incompetence of the deep veins six patients have been selected to test the possibility that the division of the popliteal vein overcomes the "bursting" type of pain on standing and decreases or eliminates postural edema. All patients have had a deep thrombophlebitis with recanalization, subsequent edema induration, and ulceration. All have been under our care for several years and were treated by compression bandages, excision of ulcers, skin grafts or saphenous vein ligation in spite of which there were recurrences. Since previous experience with femoral vein ligations gave no appreciable help division at this site was done with the following results: (1) all six patients lost their "bursting" pain on standing; (2) swelling was greatly decreased or completely abolished in every instance; (3) patches of recurrent lymphedema with ulceration were uninfluenced; this complication requiring other forms of therapy. This simple procedure may eliminate the incapacitating sequelae of postphlebitic edema in selected cases.

### 106 QUANTITATIVE STUDIES OF TREATMENT OF ACUTE CLOSED CEREBRAL INJURY BY HYPERTONIC INTRAVENOUS GLUCOSE OR SURGICAL DECOMPRESSION

C BRUCE TAYLOR M D, AND GEORGE M HASS, M D CHICAGO, ILL

Acute closed intracerebral lesions characterized by hemorrhage, necrosis and progressive edema were produced in rabbits by freezing the brain through the intact skull with an instrument cooled by expanding carbon dioxide. The dimensions and locations of cerebral lesions were controlled so that they could be reproduced in successive animals.

Volumes of cerebral damage, ranging from 13 to 32.5 vol per cent of the brain, were produced in one hundred four untreated animals. In this group, the minimum lethal quantity of damage was 9.4 vol per cent. No animal survived with damage in excess of 18.0 per cent. The average minimum lethal quantity of damage was 14.3 vol per cent. Most animals that died had a normal postoperative period of behavior. Secondary lapse into stupor was a dependable indication of eventual death within two hours.

Twenty-five animals with acute, closed, cerebral lesions were treated with intravenous injections of hypertonic aqueous solutions of glucose given continuously or by single or multiple infusions. Intravenous infusions were started shortly after the production of lesions. This therapy was of no benefit, either from the point of view of amelioration of symptoms or survival.

When surgical decompression was done with removal of bone-flaps over cerebral lesions within forty-five minutes after production (forty-seven animals) or after onset of stupor, coma, or convulsions (eight animals), symptoms were relieved and animals survived with more cerebral damage than control, untreated animals. In these animals the minimum lethal quantity of cerebral damage was 14.6 vol per cent. The maximum survivable quantity of cerebral damage was 32.7 vol per cent. The average minimum lethal quantity of cerebral damage was 21.9 vol per cent which was about 50 per cent greater than that in the untreated group (14.3 vol per cent) and in the group treated with hypertonic solutions of glucose (14.5 vol per cent). Four animals treated by contralateral surgical decompression had no increased tolerance to cerebral damage.

These data indicate that hypertonic solutions of glucose, given periodically or continuously by the intravenous route, were of no value in the treatment of acute, closed, cerebral lesions characterized by local hemorrhage, necrosis, and edema. Surgical decompression was of great benefit when done over the site of the lesion. It was concluded that surgical decompression of the type used should be evaluated as a method of treatment of patients with localized intracerebral necrosis, hemorrhage, and edema due especially to vascular disease.

## 107 FURTHER EXPERIENCE WITH BACTERIAL PYROGENS IN THE TREATMENT OF MALIGNANT HYPERTENSION

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Of eleven patients with malignant hypertension showing reversal, eight are alive without further treatment and lead useful lives. The average survival time for this group is now thirty-two months. Two patients have died of cerebral hemorrhage thirty-seven and sixteen months after treatment, and one has not been re-examined.

Six patients have been treated during the past year. Of these, three had  $T_{MPAH}^*$  values which exceeded 40 mg per minute. The blood pressure of the first patient was reduced from 175/118 to 140/84 mm Hg during six weeks of treatment. All signs of malignant retinopathy receded, the electrocardiogram and teleroentgenogram became normal, and  $T_{MPAH}$  increased from 55 to 77 mg per minute. There have been no further signs of malignancy.

The next two patients did equally well for seven and eight weeks of treatment. Papilledema receded from Grade III to Grade I, hemorrhages and exudates cleared completely in one and decreased from Grade II to Grade I severity in the other.

\*Tubular mass measured by para-aminohippurate excretion

These two patients objected to prolonged hospitalization, hence elected to discontinue treatment. One died during the second stage of lumbodorsal sympathectomy, the other shows no signs of progression of the disease.

A fourth patient had a  $Tm_{PAH}$  of only 33 mg per minute. He had Grade IV retinopathy, gross hematuria, and electrocardiographic and x-ray signs of hypertensive heart disease. He was treated for two months. The blood pressure was reduced from 204/125 to an average of 140/90. All signs of retinopathy and cardiac disease disappeared. Maximum specific gravity increased from 1.016 to 1.028.

Two patients having  $Tm$  (tubular mass) values of 25 and 9 mg per minute have done poorly. One died of cerebral hemorrhage during treatment and the other is showing signs of progressive renal failure, although the eye grounds and blood pressure now are improved.

Renal function with  $Tm_{PAH}$  values of at least 33 mg per minute continues to be the best pretreatment index of satisfactory response.

The pyrogen Pyromen is given by vein in doses large enough to cause a daily temperature rise to 102 to 104° F. The initial dose is 0.5 cc of solution containing 50 gamma of solids per cubic centimeter. Thereafter, the amount given is determined by the temperature peak of the preceding day. The daily injections should be continued as long as the blood pressure falls and signs of malignant hypertension regress.

When the blood pressure is no longer decreasing, the frequency of injections may be reduced. Over a period of two to six months there is usually a gradual rise in pressure in most of the patients and eventually hypertensive levels may prevail. However, signs of malignancy have so far not returned and the patients can be dealt with as having uncomplicated essential hypertension.

### 108 PNEUMOCOCCUS TYPES, MORTALITY, BACTEREMIA AND PURULENT COMPLICATIONS IN PRIMARY PNEUMOCOCCIC PNEUMONIA AT THE CINCINNATI GENERAL HOSPITAL, 1936-1949

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Three thousand one hundred four cases of primary pneumococcal pneumonia in patients over 13 years of age with specific typed pneumococci in sputum or blood cultures are reported here. Pneumococcus typing has been carried out as a means of specific diagnosis to evaluate various modes of therapy. The data for each year are calculated from July 1 through June 30 of the following year.

The number of cases per year decreased by gradual fluctuation from three hundred eighty-seven in 1936-37 to one hundred twenty-two in 1948-49.

Type 1 was most frequent in 1936-37, 1938-39, 1939-40, 1940-41, 1942-43, 1943-44, and 1948-49. Types 1 and 7 were equally frequent in 1944-45. Type 7 was most frequent in 1941-42, 1945-46, and 1946-47. Type 3 was most frequent in 1937-38, and Type 2 was most frequent in 1947-48.

Mortality was 36.7 per cent, 32.4 per cent, and 28.1 per cent respectively for 1936-37, 1937-38, and 1938-39 when antipneumococcal serum for certain types was the only specific treatment. However, sulfapyridine came into use in January 1939. From July 1, 1939 through June 30, 1947, sulfonamides were the mainstay of treatment. For these eight years mortality ranged from 15.3 per cent to 12.6 per cent, except that mortality was 20.2 per cent in 1940-41,

19.5 per cent in 1943-44, and 18.6 in 1944-45. In 1945-46 and 1946-47 penicillin was used for treatment if there was no response to sulfonamide, or if sulfonamide drugs were contraindicated. Penicillin was used exclusively for treatment in 1947-48 and 1948-49. Mortality was 9.7 per cent in 1947-48, and in 1948-49, when aqueous crystalline penicillin in doses of 200,000 or 300,000 units was given intramuscularly twice on the first day of treatment and once daily thereafter, mortality was 5.7 per cent.

Pneumococcus bacteremia was found in 12.9 per cent of the cases in 1940-41, and in 49.5 per cent of the cases in 1947-48. In the remaining years bacteremia was found in 16.4 to 31.8 per cent of the cases.

Empyema, meningitis, endocarditis, lung abscess, or pyarthrosis occurred in 13.2 per cent of the cases in 1937-38, and in 3.8 per cent of the cases in 1940-41. In the remaining years these complications occurred in 4.6 to 10.7 per cent of the cases.

Apparently penicillin therapy has reduced mortality, and the unchanged frequency of bacteremia and purulent complications results from the onset of these manifestations before the start of treatment.

#### 109 CONTROL COMPARISON OF NU-2206 (3-HYDROXY-N-METHYL MORPHINAN HYDROBROMIDE) WITH MORPHINE SULFATE FOR RELIEF OF POSTOPERATIVE PAIN

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In an earlier investigation of the analgesic properties of NU-2206 (3-hydroxy-N-methylmorphinan hydrobromide) the effect of this agent on the pain threshold in normal volunteer student subjects was determined. There followed a clinical trial to determine its effect in a variety of patient categories. It was found to have desirable analgesic properties with an apparently more prolonged effect than that of morphine sulfate in equivalent dosage ranges. It was found that the dosage range was from 3 to 6 mg. as compared with 10 to 15 mg. of morphine. The side effects did not appear to be severe or frequent. A limited study was made on a small number of patients who had received the drug over a prolonged period of time, and no withdrawal symptoms were observed.

Its prolonged analgesic effect appeared beneficial in those patients who required protracted relief and in whom frequent injections with shorter-acting analgesics were undesirable. Controlled study then was undertaken on ward patients undergoing surgical procedures on the General Surgical and Urological services. Those under the age of five years or in such condition as to be outside the preselected dosage range were excluded. Patients having intracranial surgical procedures also were omitted. They were divided into fifteen operation categories so that subsequent statistical evaluation as to the amount of pain associated with various types of operative procedures could be compared. In each operation category alternate patients were given NU-2206 or morphine sulfate. The medications were so dispensed as to preclude identification except by code letter.

The nursing staff was instructed to give 1 cc. for each patient or  $\frac{1}{2}$  cc. for each patient. One cubic centimeter contained either 5 mg. of NU-2206 or 10 mg. of morphine sulfate. The drugs were administered by the nursing staff to be given as required for the relief of pain not more frequently than every three hours. A special form was provided listing seven common complications

ordinarily seen in conjunction with the use of analgesic drugs. The nurses' observations were checked closely by the same member of the staff each day.

It appears, after studying 406 patients by this method that analgesic drugs are not needed in slightly more than one third of the instances. A difference in duration of action of NU 2206 and morphine sulfate was not demonstrated. NU 2206 is as effective an analgesic agent as morphine with no demonstrable increase in side actions.

## 110 SPONTANEOUS RUPTURE OF THE HEART FOLLOWING ACUTE MYOCARDIAL INFARCTION

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Cardiac rupture occurred in eight patients (7.2 per cent) of a series of 111 instances of acute myocardial infarction in patients who had been observed in the Evanston Hospital and came to autopsy during the past six years.

The eight patients were all of the white race. Their ages ranged from 51 to 86 years, averaging 68, and they were equally divided in sex. Half of the group denied prior angina; three had experienced pains from one to four months; and one who died within two days after onset of illness gave no history of anginal pain or hypertension and had considered herself in excellent health. However, there was evidence of old infarction in four of these patients.

Hypertension persisted after the onset of clinical myocardial infarction in all but one of these patients, and he belonged in the group of four who gave no prior history of hypertensive disease.

Death occurred rapidly after the occurrence of the rupture, as evidenced by the condition of the pericardial sac and lacerated tissues. Four patients showed ante mortem clotting of the blood in the pericardial sac, and one had organization of the clot, but the remaining three revealed only bright unclotted blood.

Clinical observation indicated that three expired immediately after the rupture, four within a day, and only one survived sixty hours. All eight deaths occurred within two to eleven days, average 5.8, after the clinical onset of acute myocardial infarction. The rupture occurred in the left ventricle in seven of the patients, four posteriorly and three anteriorly, while the remaining one ruptured through the right ventricle and septum at the apex.

Positive electrocardiographic evidence of acute myocardial infarction was present in all of the rupture group except one patient on whom such an examination was not possible because she expired within a half hour after admission to the hospital. The clinical diagnosis of rupture was made in six of the patients prior to autopsy.

Rupture of the heart occurred in four of these patients during defecation.

The leucocyte counts and blood sedimentation rates were consistently above normal levels in both the rupture and non-rupture groups, but there was no significant difference.

# 111 CLINICAL AND LABORATORY OBSERVATIONS IN FATTY INFILTRATION OF THE LIVER

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AND EDWARD A GALL, M D (BY INVITATION), CINCINNATI, OHIO

Fifteen patients with pure fatty infiltration of the liver demonstrated by needle biopsy were selected for study

Thirteen of the fifteen patients were admitted to the medical service for various causes among which were hematemesis, pellagra, chronic cholangitis, diabetes mellitus, chronic pancreatitis, xanthomatosis, congestive heart failure, myocardial infarction, malnutrition, and gastritis. Two patients with mycosis fungoides and discoid lupus erythematosus respectively were studied on the dermatologic service

The ages of the patients ranged from 29 to 85 years. Nine of the patients were women. A definite history of alcoholism was present in six. The diet was adequate in only six patients. An antecedent history of jaundice was obtained in two patients, both of whom had had previous cholecystectomies. Mild diabetes mellitus was present in two, while obesity was present in six patients. A history of weight loss of 10 pounds or more was obtained in ten of the subjects. Nausea and vomiting occurred in six, while anorexia was prominent in ten.

Hepatomegaly (ranging from one to five fingerbreadths) was observed in ten of the fifteen patients, while splenomegaly was present in only one instance. Edema of the lower extremities was encountered three times. Glossitis and evidences of peripheral neuritis were found in two patients.

Laboratory studies showed an elevation of the thymol turbidity in five of fourteen cases and a 3 to 4 plus cephalin cholesterol flocculation test in seven of fourteen cases. Serum bilirubin was elevated in two patients. Bromsulfalein retention was demonstrated in seven of fourteen patients. The prothrombin time was not significantly prolonged in any instance. Anemia was present in six subjects and was macrocytic in two.

It may be concluded that fatty infiltration of the liver occurs in a variety of conditions and presents variable clinical and laboratory findings, and requires needle biopsy for its diagnosis.

# 112 CHANGES IN TOLERANCE FOR GLUCOSE AND IN THE MORPHOLOGY OF PANCREATIC ISLET CELLS INDUCED BY INTRAVENOUS GLUCOSE IN DOGS

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(INTRODUCED BY HENRY R JACOBS, M D)

The possibility that damage to the islets of Langerhans can be caused by the continuance over long periods of time of glucose infusions in non-hyperglycemic amounts was the object of the present study and report. Previous investigators have shown that there is a response on the part of the blood sugar regulating mechanisms to administered glucose. It was postulated that continuous administration even in nonhyperglycemic amounts would elicit such a response in such a way that these mechanisms would have no chance to "rest" and would become exhausted.



Glucose was administered to female dogs by continuous intravenous infusion, and the blood sugar changes followed. The pancreas was examined microscopically at the end of each experiment. Short term infusions with hyperglycemic rates of glucose input have shown that such continuous infusions of glucose will cause an increase in the tolerance for glucose as evidenced by the markedly lower blood sugar seen at the end of such an infusion as compared with the blood sugar seen shortly after the infusion is started. Long term infusions (as long as forty eight days) of amounts well below the hyperglycemic glucose input were shown to cause pancreatic islet cell damage with beta cell degranulation, congestion, and hydropic degeneration, and resulted in a much poorer glucose tolerance as evidenced by much higher blood sugar levels in the hyperglycemic range at the end of a long term infusion than were caused by the same amount of glucose in the early part of a given infusion.

The results of this investigation would seem to indicate that the tolerance for glucose can indeed be increased and that such an increased tolerance stimulated by continuous intravenous glucose infusion will result in damage to the islet cells of the pancreas and eventual loss of glucose tolerance, probably due to exhaustion of the insulin producing cells in the islets of Langerhans. This same microscopic picture has been reported before as the result of hyperglycemia maintained over long periods of time by both anterior pituitary extract injections and again by injected glucose. It would seem from the results of this investigation that the damage and loss of tolerance for glucose is due not to the hyperglycemia but to the continued stimulation of the blood sugar regulating mechanisms.

### 113 THE TREATMENT OF UREMIA BY DIALYSIS ACROSS THE INTESTINAL MUCOSA

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(INTRODUCED BY N. C. GILBERT, M.D. AND GEORGE C. TURNBULL, M.D.)

The effects of dialysis across the intestinal mucosa by lavage are studied in two cases of chronic terminal uremia. The first is one of congenital polycystic kidney studied for two months. The other is a case of advanced nephrosclerosis in a woman whose right kidney had been removed surgically years ago studied for one month. The irrigating solution is lactate Ringer's with added dextrose 5 to 10 per cent. Levels of nitrogenous products in the dialysate from small bowel irrigation are equal to blood levels and the abstraction rate is inversely proportional to the rate of flow. A large inflow (priming) is required in intestinal dialysis before outflow effectively equals inflow. Irrigation of the lower ileum and colon is equally efficient and allows for control of edema. Alteration of irrigating solution concentration of glucose controls edema during irrigation. Glucose is absorbed during dialysis necessitating concentrations up to 10 per cent in the irrigating fluid to maintain isotonicity. Sodium chloride in 0.6 per cent concentration is absorbed at all levels of the intestine but sodium chloride deficient solutions will abstract as much as 0.5 Gm. per liter. In view of this an irrigating solution containing 0.45 per cent sodium chloride would seem more suitable. Sodium lactate in the dialyzing fluid in combating acidosis, hemoconcentration or dilution during dialysis did not influence blood nitrogen levels. By fastening a small bored tube four feet from the tip of a Miller Abbott tube, inflow of dialyz

ing fluid occurs through the former, and outflow through the latter, which can be allowed to remain at the ileocecal level. This was shown to cause no irritation although in place for one month. Blood nonprotein nitrogen rises rapidly in the uremic individual following intravenous administration of protein hydrolysate.

#### 114 A QUANTITATIVE STUDY OF THE SOLUBILITY OF HUMAN HEMOSIDERIN

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Previous studies of hemosiderin, isolated by diastatic chemical methods, have indicated that the product is a form of ferric hydroxide. The present studies were an attempt to define the properties of hemosiderin by less drastic methods and to compare the properties of intracellular hemosiderin with those of chemically isolated hemosiderin and synthetic ferric hydroxide.

Hepatic tissue of patients with hemochromatosis and hemolytic hemosiderosis, secondary to multiple transfusions, was used. Frozen sections of fresh tissue were dried in vacuo for qualitative microscopic studies. Frozen pulverized fresh tissue was dried in vacuo for use in quantitative studies of solubility of hemosiderin. Sections for microscopic study and small amounts of powdered tissue were exposed to the action of buffer solutions of different ionic composition (pH 12 to 11, ionic strength, 0.25 and 0.50). Ferric hydroxide and hemosiderin isolated from hepatic tissue by the usual alkaline methods were exposed to similar solutions. Solubility was determined quantitatively by analyses of iron in supernatant solution and in the undissolved residue.

The data indicated that chemically isolated hemosiderin and ferric hydroxide had similar solubility properties. Hemosiderin in the cytoplasm of reticuloendothelial cells had solubility properties similar to but not identical with those of chemically isolated hemosiderin and ferric hydroxide. Hemosiderin in hepatic parenchymal cells had solubility properties which were very different from those of chemically isolated hemosiderin and ferric hydroxide. Intracellular hemosiderin, chemically isolated hemosiderin, and ferric hydroxide were almost insoluble in isotonic saline and phosphate buffer solutions at neutrality. A large fraction (50 to 80 per cent) of intracellular hemosiderin, especially that present in the cytoplasm of parenchymal cells, was soluble in citrate buffer solutions, ionic strength 0.50, at or near neutrality, while chemically isolated hemosiderin and ferric hydroxide were almost insoluble under the same conditions. Furthermore, within a part of the range in which intracellular hemosiderin had a high solubility (pH 6.1 to 7.0), crystals of unknown composition appeared in the tissues near the sites of granules of hemosiderin. These crystals did not appear in normal liver cells and were not formed in the presence of chemically isolated hemosiderin or ferric hydroxide. The results indicate that the problem of isolating one or more forms of hemosiderin by chemical extraction at neutrality, or after crystallization in situ, can now be approached.

# 115 INFARCTION OF THE MUSCLE BUNDLES OF THE HEART

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The object of this investigation was to determine whether myocardial infarcts are confined to the muscle bundles of the heart or occur without regard to them. For this purpose the topography of seventy-two infarcts in fifty unselected hearts was plotted and compared with the known topography of the four chief muscle bundles of the cardiac ventricles (superficial sinospiral, superficial bulbospiral, deep sinospiral and deep bulbospiral muscles). It was found that all the infarcts followed definite patterns which coincided with the patterns of either one or more of the four muscle bundles. Twenty-five of the infarcts (35 per cent) in twenty-one cases (42 per cent) involved either a single superficial or a single deep bundle. The superficial muscles were involved either alone or with a deep muscle in 74 per cent of infarcts. The deep muscle bundles were involved, either alone or with a superficial muscle in 51 per cent. Depending upon the thickness of the ventricular wall involved it was possible to distinguish three types of infarcts: full thickness massive but not full thickness, and laminar infarcts. Rupture, aneurysm formation and mural thrombosis of the left ventricle were largely dependent upon the number of muscle bundles involved and the thickness of the infarct. Atrial infarcts were present in 42 per cent of the hearts. The immediate mortality of myocardial infarction was not influenced by the thickness of the infarct, the number of muscles included in it, nor by the involvement of any specific muscle.

# 116 INTERRELATION OF PTEROYLGLUTAMIC ACID AND VITAMIN B<sub>1</sub> IN INDUCED ANEMIA OF SWINE

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Swine maintained on a pteroylglutamic acid (PGA) deficient diet, with "vitamin free" casein as the source of protein, develop severe macrocytic anemia with megaloblasts in the bone marrow. Such animals respond well to PGA initially, but as the deficiency is maintained the ability to respond to PGA diminishes. Administration of purified liver extracts then evokes a response. If liver extract or a source of 'extrinsic factor' is given before PGA a poor response can be elicited once or twice after which no further response can be obtained until PGA is administered.

Two animals showed a slight response to relatively large doses of anthropein, but much less than to PGA.

One pig maintained on the PGA deficient regimen for several months was given large amounts of liver extract and later vitamin B<sub>12</sub>. There was an initial partial response, after which blood values were maintained better than previously but never became normal. Very marked macrocytosis developed and persisted. Administration of PGA then caused prompt disappearance of macrocytosis and blood values became normal.

Vitamin B<sub>1</sub> deficiency was induced in swine by a diet containing alpha protein of soy beans, supplemented with methionine as the source of protein. PGA was administered daily. Severe anemia developed in which macrocytosis was less marked and developed more slowly than in the PGA deficient animals. The marrow did not contain megaloblasts. These animals responded to crystal

line vitamin B<sub>12</sub>. In one pig, there were peculiar double and triple reticulocyte peaks, each of which occurred after a single injection of vitamin B<sub>12</sub>.

These findings indicate that PGA-deficient swine fed "vitamin-free" casein develop a deficiency of both PGA and the antianemia factor(s) of liver (solely vitamin B<sub>12</sub>?) Either PGA or liver extract will induce a hematopoietic response, but both eventually are ineffectual unless the other is present. This indicates that both factors are required for normal hematopoiesis in swine.

While vitamin B<sub>12</sub> deficiency results in anemia, a deficiency of PGA appears to be importantly involved in macrocytosis and megaloblastosis. It is suggested that in human pernicious anemia a double deficiency also exists. The PGA deficiency may be the result of dietary inadequacy or of disordered metabolism of PGA. The deficiency of vitamin B<sub>12</sub> usually is dependent on an inadequacy of "intrinsic factor" in the gastric secretion but presumably can result from dietary deficiencies of vitamin B<sub>12</sub> and other factors.

# 117 CORRELATION BETWEEN RIBOSE NUCLEIC ACID DEPLETION AND OTHER SIGNS OF LIVER DAMAGE AS INFLUENCED BY VITAMIN B<sub>12</sub>

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Disappearance of cytoplasmic ribonucleic acid compounds which are supposed to play a significant role in protein formation is one of the first signs of liver damage. Therefore, in comparison with twelve controls, the ribonucleic acid depletion (as recognized histologically) in thirty-six rats, forty-eight hours after intraperitoneal injection of a sublethal dose of carbon tetrachloride, was correlated with other signs of liver damage such as hydropic swelling of the parenchymal liver cells, increase of bromsulfalein retention, decrease of total liver proteins, and increase of histologically and chemically demonstrable fat. Chemical fractionation of total fat revealed the phospholipids remarkably constant, while all changes in the total fat were caused by variations of the neutral fat. Recent investigations had suggested that vitamin B<sub>12</sub> is important in the formation of ribonucleic acid compounds and subsequently it was demonstrated in this laboratory that the pathologic changes resulting from carbon tetrachloride (CCl<sub>4</sub>) intoxication was significantly less pronounced in rats which had previously received very large doses of vitamin B<sub>12</sub>. Therefore, forty-eight such protected rats were also included in the comparison.

In the individual animals of the intoxicated as well as the protected group, there was a parallelism in the degree of the different alterations. This parallelism was marked between the degree of fatty deposition and bromsulfalein retention. The increased bromsulfalein retention was found more related to circulatory embarrassment by the fat deposition (as judged from the presence of erythrocytes in the sinusoids) than to liver cell damage. On the other hand, there was an almost as marked parallelism between liver cell damage, ribonucleic acid depletion, and decrease in liver protein.

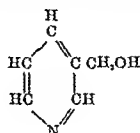
Since with small doses of CCl<sub>4</sub> ribonucleic acid depletion in the center of the lobules can be produced without other evidence of liver damage, the ribonucleic acid depletion is apparently one of the basic factors in this type of liver damage. Moreover, vitamin B<sub>12</sub> seems to inhibit liver damage by facilitating the formation of ribonucleic acid. That is probably related to the assumed role as synthesizing ribonucleases of growth factors such as vitamin B<sub>12</sub>. Their influence on protein formation in turn may control the enzyme regeneration in liver damage.

These experiences suggest the investigation of the effect of vitamin B<sub>12</sub> in different types of liver diseases. In view of the relatively large doses necessary for effect in rats, variations in vitamin B<sub>1</sub> content possibly explain the erratic results obtained with liver extracts in these disorders.

### 118 ROMIACOL—A VASODILATOR SUBSTANCE CONVERTED IN THE ORGANISM TO NICOTINIC ACID

S MARA WHITE, M D MINNEAPOLIS, MINN

Romiacol a vasodilator substance which is converted in the organism to nicotine acid is 3 pyridine methanol or B pyridyl carbinol (the alcohol corresponding to nicotine acid) and has the following structural formula



During the experimental period, the code designation for the compound was Nu 2121

Romiacol is a nonvolatile solid freely soluble in water and in alcohol and of low toxicity for animals. Administration of the human dosage to dogs for three months produced no adverse effect on weight blood formation or nonprotein nitrogen. In all animals powerful vasodilator effect on both coronary and peripheral circulation was observed.

There are several conditions in which prolonged vasodilation should be of benefit particularly in treatment but also in diagnosis. These include conditions in which coronary renal or peripheral cerebral vascular spasms are involved. Collateral circulation is of paramount importance when the vascular lumen is narrowed.

To be effective in aiding nutrition in an area with deficient vascular supply brief and evanescent dilation in collateral vessels requires frequent repetition of the dilator effect. A prolonged dilator effect is to be sought and should aid in establishing permanent dilatation of the vessels involved.

Relatively free from uncomfortable or deleterious effects and capable of causing an immediate visible flushing of the skin when given by mouth the drug can be repeated as often as desired.

This preliminary report is concerned with two conditions in which vaso spasm and vasodilation in a collateral circulation are important, the angina pectoris syndrome and peripheral vascular lesions.

In angina pectoris Romiacol has appeared repeatedly to increase tolerance for exercise and to extend the range of activity without occurrence of pain. In peripheral vascular disease the drug has been used to determine whether the total circulation of the involved parts can be improved and in cases showing improvement sympathectomy may be expected to be effective.

Cases illustrative of the foregoing conditions are cited

# 119 RAPID TREATMENT OF ACUTE GOUTY ARTHRITIS BY CONCURRENT ADMINISTRATION OF PITUITARY ADRENOCORTICOTROPIC HORMONE (ACTH) AND COLCHICINE

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RACHMIEL LEVINE, M D , CHICAGO, ILL

(INTRODUCED BY SAMUEL SOSKIN, M D )

Clinical gout appears to require two endocrine disturbances. An abnormal male sex hormone seems to regulate appearance of inherited hyperuricemia. Gout patients also appear relatively unable to meet acute relative glyccorticoid lack with a prompt increase in production. When glyccorticoid lack occurs it tends to persist and to precipitate acute gouty arthritis. Hellman and Robinson, Conn, Block, and Louis induced acute glyccorticoid lack by administering ACTH in interval gout and then withdrawing this hormone and found the procedure to precipitate acute gouty arthritis in a majority of patients. Their observations have been confirmed.

ACTH, given during acute gouty arthritis, repairs the glyccorticoid lack and rapidly terminates the episode. However, given alone, ACTH is not satisfactory therapy for acute gouty arthritis. In most patients the relative glyccorticoid deficiency which follows hormone withdrawal precipitates a renewed attack shortly after withdrawal.

Colchicine, given during or immediately after treatment of acute gouty arthritis with ACTH, effectively overcomes the tendency of ACTH withdrawal to precipitate a renewal of the attack. Single 50 mg doses of ACTH (Aimour) have effectively terminated most attacks within four hours. Occasionally a second or third dose has been required at six-hour intervals after the first. Although a number of the episodes treated had been prolonged and resistant to previous therapy, no ACTH-treated attack has persisted more than twenty-four hours after hormone therapy was begun.

Colchicine administration has been begun either with ACTH administration or four hours following the last dose of ACTH. All patients have been colchicized to tolerance and maintained on daily subtolerance doses of colchicine for at least two weeks. No patient who has received combined ACTH-colchicine therapy has had even a minor recurrence of acute gouty arthritis within one month of treatment.

Concurrent administration of ACTH and colchicine appears to be the most rapid and effective available treatment for acute gouty arthritis. In view of the relative scarcity of ACTH, the small amounts of this hormone which are required constitute a particularly desirable feature.

# 120 EFFECT OF HYPERVENTILATION ON THE HEMORESPIRATORY EXCHANGE IN NORMAL PERSONS, PATIENTS WITH PULMONARY EMPHYSEMA, AND PATIENTS WITH CARDIAC DYSPNEA

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(BY INVITATION), AND RICHARD V EBBERT, M D  
MINNEAPOLIS, MINN

Respiration aids in maintaining a normal blood pH by regulating the tension of carbon dioxide in the alveoli and hence in the arterial blood. Hyperventilation in normal persons produces alkalosis by sudden lowering of the alveolar and blood pCO<sub>2</sub>.

The purpose of this study was to determine the response to voluntary hyperventilation of patients with dyspnea secondary to heart disease or chronic pulmonary emphysema. In twelve normal persons, fourteen cardiac patients and twelve patients with pulmonary emphysema the total lung volume and its subdivisions, the minute volume, oxygen uptake, CO elimination, arterial blood pH, CO content and oxygen saturation were measured in the basal state and after two minutes of voluntary hyperventilation. The pCO of arterial blood was estimated from the nomogram of Singer and Hastings.

In the group of patients with emphysema the residual air was markedly increased with a corresponding reduction in vital capacity, the total lung volume being normal. Patients with heart disease showed a reduction in vital capacity and total lung volume with a normal residual air.

Mean values before and after hyperventilation in normal persons and patients with cardiac dyspnea were very similar. In the group of patients with emphysema the initial pCO<sub>2</sub> was elevated and the oxygen saturation was diminished, the value for the former being 50 mm Hg and for the latter 83 per cent. The blood pH was normal as the result of a compensatory increase in plasma bicarbonate. Voluntary hyperventilation was relatively ineffective in this group and did not significantly alter the initial values.

Hence patients with cardiac dyspnea like normal persons have ventilatory control over blood pH. Inability of patients with emphysema to increase alveolar ventilation effectively by maximal effort results in serious impairment of ventilatory regulation of blood pH. This defect renders these patients particularly vulnerable to metabolic and respiratory acidosis and is a constant threat to life.

## 121 A SINGLE SCALE ABSOLUTE READING EAR OXIMETER

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(INTRODUCED BY C. F. CODE, M.D.)

Two modified oximeter earpieces were calibrated on the basis of their responses on empirical optical filters using the single scale circuit described by Wood and Geraci.

Earpiece 1 was used to determine arterial oxygen saturation on thirty-three ears of eighteen normal subjects. Thirteen were white and five were Negroes. Ages ranged from 2 to 50 years. The average saturation obtained when the subjects were breathing air was  $96.7 \pm 0.3^*$  (93-101) per cent and  $99.4 \pm 0.4$  (94-106) per cent when breathing oxygen.

Earpiece 2 was used on twenty-four ears of seventeen normal subjects. Twelve were white, two were Chinese, and three were Negroes. The average arterial saturation was  $96.7 \pm 0.3$  (94-100) and  $99.7 \pm 0.5$  (96-104) per cent when the subjects were breathing air and oxygen respectively.

Eighteen simultaneous Van Slyke and oximetric determinations of arterial oxygen saturation were made on six patients with congenital heart disease during supine rest breathing air or oxygen, standing and walking. The per cent arterial oxygen saturation by Van Slyke analysis of radial artery blood averaged 85 and ranged from 39 to 100 per cent. The standard deviation of the differences between simultaneous Van Slyke and ear oximeter determinations in these hypoxemic patients was 4 per cent.

\*The number following the  $\pm$  sign is the standard error of the mean  $N = 23$ .

The instrument can be A C or battery operated, is compact, relatively inexpensive, simple to operate, and is apparently accurate enough to warrant its use for most clinical estimations of arterial oxygen saturation

## 122 STUDIES OF HEMAGGLUTININS IN CONGENITAL AND ACQUIRED HEMOLYTIC ICTERUS

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WITH THE TECHNICAL ASSISTANCE OF CHARLES A CUNNINGHAM, B S

The immunohematologists studying the Rh mechanisms developed a series of techniques for demonstrating incomplete (blocking) antibodies which have proved useful in investigating other hemolytic anemias. To the Coomb's, "developing" and "conglutination" tests, Wheeler, Lohby, and Scholl have recently added a technique,\* previously suggested by Bunnet, Pickels, and Quilligan, using enzyme-treated normal red blood cells for detecting incomplete Rh antibodies in sera. They found the technique a simple, reliable, sensitive, inexpensive, and rapid screening test for demonstrating incomplete Rh antibodies in sera of Rh-negative women. We have added this to the battery of techniques in the study of congenital and acquired hemolytic icterus. The following observations have been made: (1) 255 normal sera gave no agglutination with trypsinized red blood cells. (2) In twenty cases of acquired hemolytic icterus, one or both tests were positive in sixteen cases, fourteen with trypsinized red blood cells, fourteen with Coomb's serum. (3) In twenty-one cases of congenital hemolytic icterus, one or both tests were positive in eight cases, six with trypsinized red blood cells, four with Coomb's serum.

It is concluded that (1) the trypsinized red blood cell test is a valuable adjunct for demonstrating incomplete antibodies in acquired and congenital hemolytic icterus. (2) In acquired hemolytic icterus the avidity and titer of the trypsinized red blood cell test are generally greater than with the Coomb's test, however, they run a closer parallel in acquired than congenital types. (3) The Coomb's test, contrary to recent claims, has not been a reliable means of differentiating acquired and congenital hemolytic icterus thus far in our hands.

## 123 CAVITY POTENTIALS OF THE HUMAN VENTRICLES

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(INTRODUCED BY ROY W SCOTT, M D)

The intracavitary potentials of the human left ventricle have been studied by retrograde arterial catheterization. In eight men, 48 to 74 years of age, the left ventricle was entered successfully in six, while in two the tip of the

\*The trypsinized red blood cell technique (modified after Wheeler) 1 ml of washed (three times in cold normal saline) packed type O red blood cells is added to 15 ml of a 0.1 per cent trypsin (Difco 1:250 activity trypsin) saline (buffered to pH 7.2) solution. Suspension incubated thirty minutes 37° C with frequent agitation. Centrifuge, remove supernatant and wash enzymized red blood cells three times with cold saline. Make a 2 per cent cell suspension in saline. Suspension is checked for nonspecific agglutination with normal sera.

Serial dilutions of the patient's serum are made with 0.1 ml of serum and 0.1 ml of saline. Two drops of the above suspension of trypsinized red blood cells are added. Incubate at 37° C thirty minutes and read for agglutination.

Normal sera are run as controls.



catheter electrode was obstructed by the aortic valve. All patients had aortic insufficiency, with an average pulse pressure of 98 mm Hg. The right ventricle potentials were obtained by venous catheterization.

In the region of the upper part of the interventricular septum, curves from the right ventricle showed a small R wave and a deep S wave, in the corresponding region of the left ventricle the complex was of the QS variety, with an initial slurring on the downward limb corresponding to the R wave of the right ventricular lead. In no case was a positive deflection recorded in the left ventricle in this region. These findings are consistent with the current belief, proposed by Wilson, that the upper part of the interventricular septum is depolarized from left to right.

In one case, however, curves from the apex of the left ventricle showed a definite R wave and a deep S wave. The origin of this R wave is obscure and may be due to (1) depolarization of the lower septum from right to left, or (2) to the tip of the catheter electrode being situated in the apex which would be "facing" the wave of depolarization passing down the Purkinje system of the left ventricle.

Premature beats were most frequently produced when the catheter first entered the cavity of the left ventricle in the upper septal region. In several cases runs of ventricular beats occurred for two to four seconds, spontaneously disappearing when the catheter was withdrawn or passed forward toward the apex. Premature beats originating from the endocardium of the left ventricle produced a wide QS complex, and usually a positive QRS complex in the right and left arm unipolar leads and a negative QRS complex in the left foot unipolar lead.

Tracings were also obtained above the aortic ring and in the arch of the aorta. The ventricular complexes resembled those of the right arm unipolar lead. Thus in a patient with ST elevation in aVr, and depression of the ST segment in V<sub>3,4</sub> and in aV, the ST elevation in the lead from the arch of the aorta indicated that essentially cavity potentials were being recorded in aVr and aortic leads. However aVr did not resemble the intracavitary lead in all cases. One patient with marked left axis deviation and left ventricular hypertrophy had positive aVr and aV<sub>1</sub>, but the cavity leads of the right and left ventricles had net negative values.

## 124 A STUDY OF PULMONARY HEMODYNAMICS DURING PNEUMONECTOMY

### A PRELIMINARY REPORT

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(INTRODUCED BY ROY W. SCOTT, M.D.)

Cournand has studied pulmonary hemodynamics before and after pneumonectomy in human beings, but there are no published data on the changes occurring during the actual operative procedure. We have studied five patients undergoing pneumonectomy by the technique of right heart catheterization during the operation, when a sudden burden may be expected to be thrown on the vascular bed of the remaining lung following ligation of the opposite vessel. A catheter is placed in the pulmonary artery opposite to the one to be ligated sixty minutes before the induction of anesthesia. Basal cardiac output and initial pressures are recorded and subsequent pressure

levels are determined at fifteen-minute intervals throughout the entire procedure, including the period immediately following ligation of the pulmonary artery. Intra-arterial brachial pressure, phase of respiration, pulmonary arterial pressure, and the electrocardiogram are recorded simultaneously on a six-channel oscillograph described elsewhere. In two patients who had lesions which were not resectable, pulmonary artery pressure measurements were obtained after temporary ligation of one pulmonary artery.

We have demonstrated a consistent average rise in pulmonary arterial pressure of 50 per cent above the basal level in all instances immediately following ligation. Pulmonary artery pressures dropped to near basal levels by the end of the operation in the three patients in whom resections were done. This took an average time of forty-five minutes. These findings agree with the recent work of Long and associates in dogs.

We believe that the preoperative measurement of pulmonary arterial pressure is an important adjunct in the evaluation of the patient's suitability for pneumonectomy. This immediate rise in pulmonary arterial pressure, with the sudden load thrown on the right ventricle, may be responsible for unexpected deaths which occur after ligation of a pulmonary artery.

Scott and Zimmerman have shown that elevated pulmonary arterial pressures due to a variety of causes may be lowered by aminophylline. The effect of this drug administered prior to ligation of the pulmonary artery is under investigation.

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